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Anterior-Posterior Patterning of the Avian Neuraxis

Amanda Nadia Albazerchi

PhD. Developmental Biology

University College London

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Nieuwkoop and Nigtevecht's (1954) model of anterior-posterior patterning of the neuraxis states that the naïve ectoderm first receives an 'activation' signal giving it an anterior, neural character and subsequently a 'transformation' signal that progressively caudalises the axis resulting in the full rostro-caudal pattern. Stern (2001) proposed a modification to this model that divides the first step into two: a transient 'activation' step and a subsequent 'stabilisation' step.

The hypoblast has been implicated as the transient inducer of a pre-neural, pre-forebrain state. In this thesis, the molecular nature of this induction is investigated by grafting the hypoblast into the area opaca to look at the induction of the genes *Sox3*, *Otx2*, *ERNI* and *Cyp26A1*. It was found that FGFs recapitulate the induction of the first three markers and retinoic acid (RA) can induce *Cyp26A1* whilst loss of function experiments show that both FGF and RA are required for hypoblast-mediated induction.

In the epiblast, these induced genes are maintained as the future forebrain develops. Potential stabilising signals were tested by combining hypoblast grafts with cells secreting various proteins. By antagonising Wnts and/or BMPs and/or Nodal, *Sox3* and *ERNI* can be maintained, whilst *Otx2* maintenance requires combined Wnt- and BMP-inhibition, but the definitive neural marker, *Sox2*, is never induced. This suggests that a further 'neuralising' step might be required.

Unlike regions of the epiblast fated to form head structures, the cells that will contribute to the remainder of the neuraxis reside within a small population of progenitors near the node. This indicates that a different mechanism might be responsible for patterning more caudal regions mediated by a qualitative or quantitative mechanism. To test this, secondary axes were generated by grafting progressively older donor nodes but the patterning of these ectopic axes suggests that the node might caudalise in conjunction with the pre-somitic mesoderm (PSM). Indeed, homotopic PSM grafts between different staged embryos do affect the neural tube boundary of *Hoxb9*. PSM can caudalise the neurectoderm, an effect that increases with age of the donor and decreases with the age of the PSM cells.

An interesting conclusion is that some of the same signals are responsible both for the initial activation stages *and* for the subsequent transformation steps. This

highlights the importance of timing as to the response of a particular cell to a particular signal.

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Contents

Figures and Tables

Chapter 1: General Introduction

1.1 Introduction

Neural induction of the vertebrate embryo and the anterior posterior patterning of the neuraxis have been studied for more that a century. Induction has been defined as "...an interaction between one (inducing) tissue and another (responding) tissue, as a result of which the responding tissue undergoes a change in its direction of differentiation" (Gurdon, 1987). The principal tissue deemed to be responsible for the processes of neural induction and patterning is the organizer. The discovery of the 'Spemannn Organizer' by Spemannn & Mangold in 1924 was made when the dorsal blastopore lip from an amphibian *Triton cristatus*, *T.taeniatus* or *T. alpestris* gastrula stage embryo was grafted onto the ventral side (presumptive epidermis) of a differently pigmented host of the same stage. A secondary neural plate formed which developed into a neural tube, delayed only slightly when compared to the primary axis. The majority of cells comprising this axis were of host origin, indicating that the secondary axis had been induced in the host by the donor tissue. The secondary axis was also patterned rostro-caudally indicating that the organizer has the ability to induce and pattern a complete axis (Spemannn and Mangold, 1924). This finding resulted in a wealth of experiments and models to explain the phenomenon.

1.2 Brief description of the early stages of development of the avian embryo

1.2.1 Pre-Streak Stages

At the time of egg laying, the embryonic epiblast, the area pellucida, is a single layer of cells. The surrounding extra-embryonic area opaca is multilayered as the epiblast here is attached ventrally to large, yolky cells. The region of epiblast between the area opaca and area pellucida is the marginal zone.

At stage X [stages prior to the appearance of the primitive streak are denoted by Roman numerals I-XIV (Eyal-Giladi & Kochav, 1976)], at the boundary between this region and the area pellucida, there is a crescent-shaped ridge: Koller's sickle (or Rauber's sickle) which marks the side at which the future posterior end of the embryo will form (Callebaut & Van Nueten, 1994; Koller, 1882). However, the cells residing here at this stage are mainly dorsal (future organiser) and prospective

forebrain (Hatada and Stern, 1994; Bachvarova et al., 1998; Foley et al., 2000; Streit et al., 2000), rather than presumptive tail. By stage X, islands of yolky cells are present on the ventral side of the area pellucida, perhaps through polyingression occurring before laying (Eyal-Giladi, 1984; Fabian & Eyal-Giladi, 1981; Kochav, Ginsburg & Eyal-Giladi, 1980; Peter, 1938). Between stage X and XIII these cells gradually coalesce into a layer. It is most likely that the hypoblast cells join together by flattening to form a loose but coherent layer (Vakaet, 1970; Eyal-Giladi & Kochav 1976; Stern, 1990) starting at the posterior end and then gradually spreading as a sheet of cells in a posterior-to-anterior direction to cover the entire area pellucida. Half of the area pellucida is covered by this layer at stage XII and all of it by stage XIII. This layer is termed the 'hypoblast'.

The hypoblast is then displaced anteriorly starting at stage XIV by the formation of a second primitive endoderm layer emerging behind the hypoblast: the endoblast (Vakaet 1970). Several markers expressed in the hypoblast are not expressed in the endoblast and hence the two can be distinguished molecularly [*goosecoid, Hex, Hesx1/Rpx, Cerberus/Caronte, Otx2* and *Crescent* (Bachvarova et al., 1998; Foley et al., 2000; Bertocchini & Stern, 2002)].

Between stages X and 3 (Arabic numerals are used from stage 2 [early streak] onwards following the staging system of Hamburger and Hamilton, 1951), as the primitive streak starts to form and extend towards the centre of the embryo, there is a great deal of cell movement in the epiblast, described as 'Polonaise' (Gräper, 1929). These movements completely rearrange the positions of the prospective territories. Prospective dorsal regions, initially in a posterior, medial position, move to the centre being replaced by more lateral cells that have more ventral fates. This dramatic rearrangement is illustrated in the diagram below (Fig.1.1)

The movements of the hypoblast, forming a sheet of cells and moving from posterior to anterior, is related to the midline extension in the epiblast which occurs at a similar speed to the hypoblast spreading (Hatada & Stern, 1994). Rotation of the hypoblast causes a concordant change in the position of the prospective forebrain in the embryo proper, which is due to a redirection of the epiblast cells (Waddington, 1930; Waddington, 1932; Waddington, 1933; Foley et al., 2000). Also see Fig 2.1.

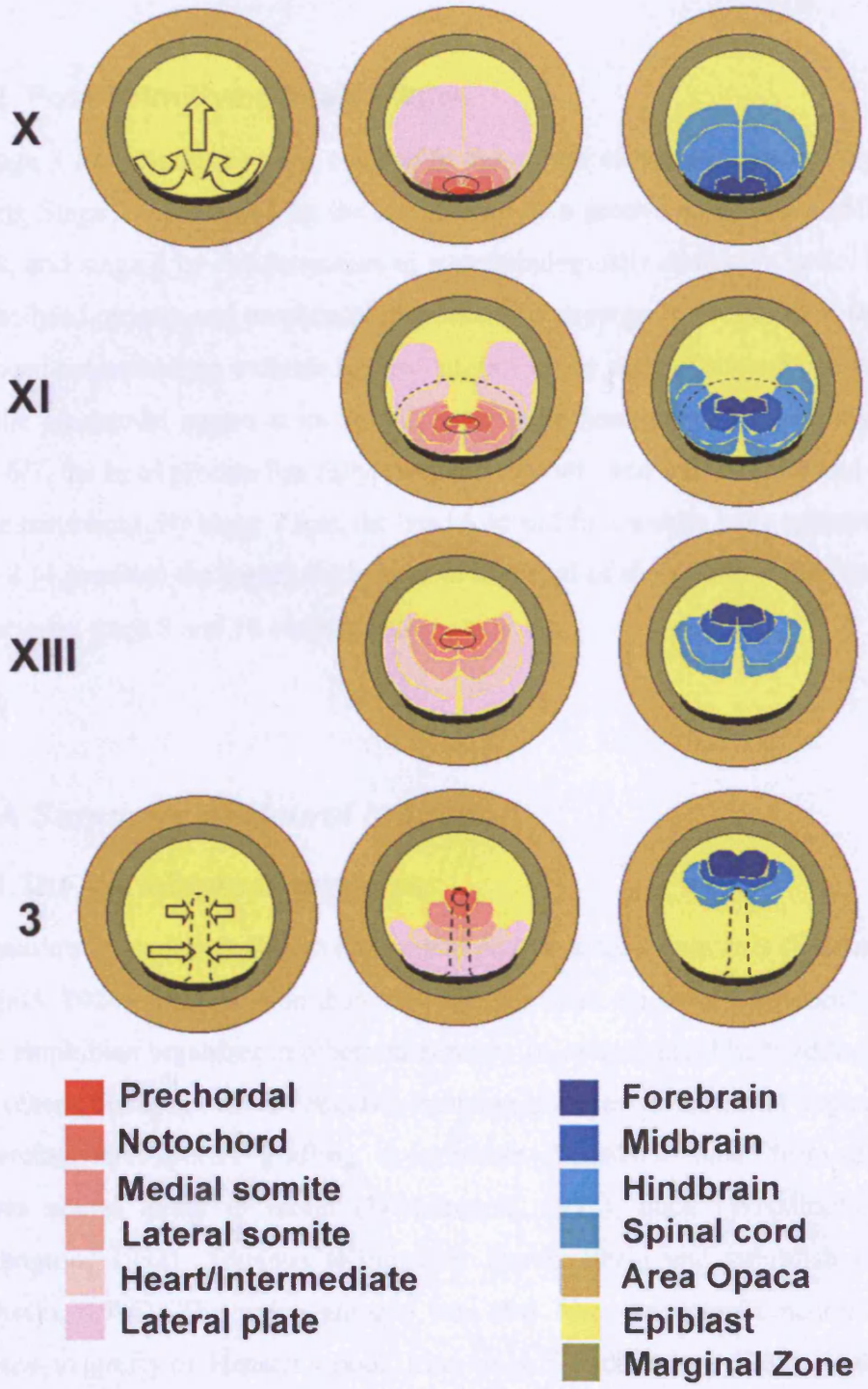


Figure 1.1 This cartoon illustrates the polonaise movements of the epiblast at pre- streak stages and during gastrulation, constructed from fate maps, when there is still only one embryonic layer.
The left-hand column shows Polonaise movements of the epiblast before streak formation and convergence of the epiblast to the streak, strongest posteriorly, during gastrulation. The middle column of picutres shows the territories that will give rise to the mesodermal tissues and the final column inducates prospective neural plate regions.
Taken from Stern, 2004.

1.2.2. Post-Primitive-Streak Stages

At stage 3 the primitive streak extends to the centre of the embryo but there is no groove. Stage 3+ is defined by the appearance of a groove along the middle of the streak, and stage 4 by the formation of a morphologically definable node. By stage 4+, the head process and prechordal mesendoderm emerge from the tip of the streak. The head mesendoderm extends further anterior to the node at stage 5 and is divided into the prechordal region at its tip and the chordal head process more caudally. At stage 6/7, the head process has fully extended rostrally and will be followed caudally by the notochord. By stage 7 both the head fold and first somite have appeared and at stage 8 (4 somites) the neural folds meet at the level of the midbrain. Seven somites characterise stage 9 and 10 somites define stage 10.

1.3 A Summary of Neural Induction

1.3.1 The Vertebrate Organizers

Spemannn's Organizer is able to induce and pattern a secondary axis (Spemannn and Mangold, 1924). The question then was whether there exists an equivalent structure to the amphibian organizer in other vertebrates. To investigate this, Waddington, and later others, investigated the relative inducing abilities of potential organizers by performing inter-species grafting experiments. 'Hensen's node' from the chick induces neural tissue in rabbit (Waddington, 1937), duck (Waddington, 1930; Waddington, 1932), Xenopus (Kintner & Dodd, 1991) and zebrafish (Hatta & Takahashi, 1996). The avian embryo can also form an ectopic neural plate in response to grafts of Hensen's node from duck (Waddington, 1930; Waddington, 1932), rabbit (Waddington, 1934; Waddington, 1936a; Waddington, 1937) (Knoetgen et al., 2000) and mouse (Zhu et al., 1999; Knoetgen et al., 2000) embryos. Waddington's (1930, 1932, 1934, 1937) xenograft experiments showed that avian and mammalian nodes can indeed, when grafted into host tissue, result in the formation of a secondary axis. However, he could not distinguish induction from recruitment and patterning of host cells. This problem was initially investigated by Waddington (1934) and later thoroughly addressed by Gallera (1964; 1969; 1970;

1971) who performed a series of grafting experiments of the node into different regions of the area opaca and area pellucida. He determined that the area opaca is competent to respond to neural inducing signals from the node and to produce a secondary axis. Cells in the area opaca were considered naïve; not having received any prior inductive signals from the endogenous organizer, leaving no doubt that the secondary axis was the result of an induction (Gallera, 1969; 1970; 1971). This work was furthered more recently when it was shown that a young avian node from a stage 3-4 donor can induce a fully regionalised secondary axis when grafted into the area opaca (Dias and Schoenwolf, 1990; Storey et al., 1992). Nodes taken from older donors (stage 5-6) lose the ability to induce the most anterior structures, generating only a posterior axis that tends to contain more graft-derived cells (Dias and Schoenwolf, 1990; Storey et al., 1992). This shows that the avian node is functionally homologous to Spemannn's organizer and that the window of its full inducing activity is limited. In the mouse the situation is less clear. The mouse node is able to induce and pattern a secondary axis (Beddington, 1994) but this axis lacks anterior structures much like those generated by older avian nodes. A transient organiser, termed the MGO (mid-gastrula organiser), which is found at mid-streak stages, is able to induce and pattern a secondary axis expressing the anterior neural marker, *Otx2* (Kinder et al., 2001). This will be discussed further below. In teleosts, it was discovered that if the embryonic shield region is grafted into an ectopic position in a host it will induce a secondary axis. These experiments were performed originally in *Fundulus* and *Perca* (Oppenheimer, 1936) and in trout (Luther, 1935). More recently, careful analyses on the inductive properties of the shield region were performed (Shih and Fraser, 1996; Saúde et al., 2000) revealing that when grafted into the ventral germ ring, the shield can induce a complete secondary axis.

1.3.2 The Default Model for Neural Induction

It is clear from the experiments mentioned above that the organizer or node can induce a neural state in naïve ectoderm but the precise molecular nature of neural induction is, as yet, unclear. Many experiments, predominantly in *Xenopus*, have lead to the 'default model' for neural induction (Hemmati-Brivanlou & Melton, 1997). The findings that led to this model include the following: *BMP4* is initially expressed in the entire ectoderm in the early gastrula and later clears from the prospective neural plate (Fainsod, Steinbeisser & De Robertis, 1994). BMP

signalling was inhibited experimentally in several ways by different groups. A dominant negative form of the "activin" receptor (Hemmati-Brivanlou & Melton, 1992; Hemmati-Brivanlou & Melton, 1994); a dominant negative BMP2/4-receptor (Graff et al., 1994; Suzuki et al., 2994); and inhibitory forms of SMAD proteins (von Bunoff and Cho, 2001) will all antagonise the BMP signalling pathway to promote dorsal fate. It was also found that several proteins inhibit BMP signalling by binding directly to BMP. These include Noggin (Smith & Harland, 1992; Lamb et al., 1993; Smith et al., 1993), Chordin (Sasai et al., 1994; 1995) and Follistatin (Hemmati-Brivanlou et al., 1994). Inhibition of BMP signalling by each of these factors resulted in the formation of ectopic neural tissue. Furthermore, Noggin (Smith & Harland, 1992; Lamb et al., 1993; Smith et al., 1993), Chordin (Sasai et al., 1994; 1995) and Follistatin (Hemmati-Brivanlou et al., 1994), as well as other BMP antagonists like Cerberus (Bouwmeester et al., 1996; Belo et al., 1997), are expressed in or near the organizer. This suggested that during normal development BMP must be antagonised for neural induction to occur. This proposal was supported by evidence that over-expression of BMP resulted in a ventralisation of the embryo (Dale et al., 1992; Jones et al., 1992), including the absence of neural tissues and over-representation of epidermal fates (Hawley et al., 1995; Wilson & Hemmati-Brivanlou, 1995). Further evidence for the role of BMPs in inhibiting neural induction came from cell dissociation experiments. BMP is a secreted factor and when gastrula-stage animal caps from *Xenopus* embryos are dissociated briefly before reaggregation they will form neural tissue (Born et al., 1989; Godsave & Slack, 1989; Grunz & Tacke, 1989; Saint-Jeannet, Huang & Duprat, 1990; Sato & Sargent, 1989) which is prevented by the addition of BMP4 effectors (Suzuki et al., 1997a; Suzuki, Ueno & Hemmati-Brivanlou, 1997b; Wilson et al., 1997). These results suggest that, when cells are dissociated, extra-cellular BMP is lost allowing cells to develop according to a "default", neural fate. All this evidence was used to derive the 'default model' of neural induction, which proposes that in the absence of any external signals, ectodermal cells have an intrinsic tendency to become neural instead of epidermal. In order to prevent the default neural pathway and to promote epidermal fate, an antagonist of neural induction is required which is BMP (Hemmati-Brivanlou & Melton, 1997) (Fig1.2).

Although there are convincing data supporting the 'default model' in *Xenopus*, it seems like it might not be the whole story. Indeed, in the chick, the default model of

neural induction appears insufficient to explain the data. The expression patterns of BMPs and their antagonists do not accurately match those in the frog (Streit et al., 1998). *BMP4* and *BMP7* are only weakly expressed in the ectoderm before neural induction and by stage 4 their expression increases at the border of the neural plate. Likewise, *noggin* and *follistatin* are not expressed at the correct stages to play the role suggested in the 'default model' and *chordin* continues to be expressed in the node after it is no longer capable of inducing neural tissue (Streit et al., 1998). In addition, blocking BMP is not enough to induce neural tissue in competent epiblast (Streit et al., 1998; Streit & Stern, 1999; Linker & Stern, 2004) and the dissociation of epiblast cells results in muscle differentiation rather that neural fate (George-Weinstein et al., 1996). In the fish and mouse there are also problems with the model. Zebrafish expression patterns show that *follistatin* and *noggin* are not expressed in the correct place to suggest their inhibition of BMP permits neural induction (Bauer et al., 1998). The zebrafish *chordin* mutant, *chordino* (Hammerschmidt et al., 1996a; Hammerschmidt et al., 1996b; Kishimoto et al., 1997; Schulte-Merker et al., 1997; Bauer et al., 1998), and mouse mutants null for *noggin*, *chordin* or the double mutants all still develop neural plates (Brunet et al., 1998; McMahon et al., 1998; Bachiller et al., 2000). These data suggest that BMP inhibition might not be sufficient to explain neural induction. Other molecules are now thought to be involved which include FGFs and Wnts.

In the ascidian *Ciona*, FGF signalling through the MEK pathway is both required and appears sufficient for neural induction rather than BMP inhibition (Darras and Nishida, 2001; Hudson and Lemaire, 2001; Kim and Nishida, 2001; Bertrand et al., 2003; Hudson et al., 2003). There is growing evidence in vertebrates that FGF must also play a role in neural induction. Suramin, which inhibits FGF (amongst other proteins), can block neural induction (Grunz, 1992), as can SU5402, a more specific FGF inhibitor (Delaune et al., 2005). XFD (a dominant negative form of the FGF receptor 1) can prevent Chordin and Noggin from neuralising ectoderm (Launay et al., 1996; Sasai et al., 1996). In addition, even the process of cutting the animal cap from *Xenopus* activates the MAP kinase pathway (LaBonne & Whitman, 1997), which suggests that FGFs might be playing a previously unconsidered role in animal cap experiments that implicated BMPs. Recent evidence suggests that cell dissociation of *Xenopus*, in addition to diluting extracellular BMPs, also activates the MAPK pathway and this results in the phosphorylation of SMAD1 (the intracellular

effector of BMP) preventing effective BMP signalling (Kuroda et al., 2005). FGF has been shown to induce neural tissue directly in some situations (Lamb & Harland, 1995; Rodriguez-Gallardo et al., 1997; Storey et al., 1998; Alvarez et al., 1998; Barnett et al., 1998; Wilson et al., 2000; Hardcastle et al., 2000; Hongo et al., 1999; Hudson et al., 2003; Ishimura et al., 2000; Kim & Nishida, 2001;) although this has been shown to be insufficient for direct neural induction by itself in other studies in chick and *Xenopus* (Streit et al., 2000; Linker and Stern 2004; Delaune et al., 2005).
It has been suggested that FGF alone can block the BMP pathway and permit neural induction in prospective neural plate cells whereas inhibition of Wnt signalling is also required to block BMP in prospective epidermal cells in chick embryos (Wilson et al., 2000; 2001). An early positive role has been proposed for Wnt signalling for neural induction but it is then necessary to block it for the process to continue (Sokol et al., 1995; Baker et al., 1999; Wessely et al., 2001; Bainter et al., 2001; Wilson et al., 2001; Kuroda et al., 2004). The ectoderm of mouse mutants for *Wnt3* does not undergo neural induction (Liu et al., 1999). Injection of *dishevelled* (required for Wnt signal transduction) mRNA into prospective ventral mesoderm cells results in a dorsalisation of the axis whilst injection into ectodermal cells induces anterior neural differentiation (Sokol et al., 1995). It was also shown that Wnt8 can inhibit *BMP4* at gastrulation stages in *Xenopus* (Baker et al., 1999) and that β-catenin has a role in activating BMP antagonists, such as Chordin, in the dorsal side of the embryo (Wessely et al., 2001). Furthermore, the presumptive neural plate in the blastula can undergo neural differentiation when cultured in the absence of mesoderm but this requires the expression of β-catenin to induce *chordin* and *noggin* (Kuroda et al., 2004). These data indicate that in early development the promotion of dorsal structures and neural induction could be effected through BMP inhibition and the activation of BMP antagonists by Wnt signalling. This theory can be reconciled with the results in the chick (Wilson et al., 2001) if Wnt is considered to have a very early role in neural induction and subsequently must be blocked.
Recent studies in *Xenopus* and zebrafish extend the idea of the 'default model' to include signalling by FGF and IGF (insulin-like growth factor) in the regulation of BMP at the level of SMAD1 phosphorylation (Pera et al., 2003; Cha et al., 2004; Londin et al., 2005). IGF was previously shown to be a neural and head inducer in *Xenopus* (Pera et al. 2001) and IGF, FGF and Chordin can expand the neural plate when injected into the blastocoele cavity of the *Xenopus* embryo. Combinations of

these proteins have additive (synergistic) activity when co-injected to expand this region and it was also found that Chordin requires FGF and IGF signalling to act as a neural inducer (Launay et al., 1996; Kretschmar et al., 1997; Sasai et al., 1996; Pera et al., 2003). The mechanism by which these pathways might interact was investigated. SMAD1 becomes phosphorylated upon activation by the BMP receptor promoting nuclear translocation and transcription (Massagué and Chen 2000). SMAD1 can also be phosphorylated by MAPK in the central linker region, which causes cytoplasmic localisation and inhibition of transcription: the opposite effect of BMP-mediated phosphorylation (Kretzschmar et al. 1997). To test the importance of MAPK phosphorylation of SMAD1 in neural induction, two constructs were created: the first expressed *Smad1* and the second a form of *Smad1* that had a mutation in the MAPK phosphorylation sites to make it insensitive to this process. The first construct resulted in a mildly ventralised phenotype when injected into the animal pole of 4-cell stage *Xenopus* embryos. However, the mutant form caused a very severe ventralised phenotype with embryos lacking a head and most of the CNS and having ventralised mesoderm. This suggested that to cause a strong ventralisation it is important to block MAPK phosphorylation of SMAD1 but to allow phosphorylation by BMP. It was further shown that the mutant form of SMAD1 was much more potent in inhibiting neural induction and neuronal differentiation than the wild-type form. This shows that MAPK is required to phosphorylate SMAD1 to allow neural induction and *in vitro* and *in vivo* experiments showed that FGF8 and IGF can phosphorylate SMAD1 via the MEK/ERK MAPK pathway (all from Pera et al., 2003). In the mouse mutant for the central linker region of *Smad1* a much less severe phenotype was observed and there are distinct phenotypes observed for this mutation and in a mutant in which the BMP phosphorylation site is disrupted (Aubin et al., 2004). The authors suggest that the overall amount of linker-region found in SMAD1, SMAD5 and SMAD8 might have to be manipulated to discover the full effect of the role of MAPK phosphorylation of SMADs in neural induction. However, it also indicates that the modulation of SMAD1 at neural induction stages might not be a simple switch between phosphorylation by MAPK and BMP. Consistent with this, non-neural ectoderm can be prevented from acquiring an epidermal fate by inhibition of SMAD1 or BMP but it does not become neural without additional low levels of eFGF (Delaune et al., 2005) suggesting that FGF has further roles in neural induction than blocking BMP signalling.

Recently, in the chick, the roles of FGF and BMP-inhibition in neural induction have been re-investigated (Linker & Stern, 2004). It was shown that BMP inhibition is not sufficient for the early expression of *Sox3* but is required for later expression of *Sox3* as well as for *Sox2*. This suggests that BMP inhibition is only necessary for the formation of definitive neural tissue. This is more consistent with the expression pattern of *BMP4* and *BMP7* at the boundary of the neural plate at the end of gastrulation when definitive neural tissue starts to be formed in the chick (Streit et al., 1998). BMP antagonists *Smad6*, *noggin* and *chordin*; FGF2, FGF3, FGF4 or FGF8 and Wnt antagonists were applied to the area opaca either alone or in combination; none of the combinations were sufficient to induce the expression of the definitive neural marker, *Sox2* (Linker & Stern, 2004).

There is still some support for the 'default model' in *Xenopus* however. Recently, three of the BMP antagonists, Noggin, Chordin and Follistatin, have been inhibited, either alone or in combination, using morpholino injection into *X. tropicalis* (Khokha et al., 2005). When one of these antagonists is inhibited, the resulting phenotype is that of a mildly narrowed neural plate, or no effect at all on the neural plate in the case of follistatin. If two BMP antagonists are inhibited this phenotype is exacerbated, so that the neural plate is reduced further. However, when Khokha et al. (2005) inhibit all three antagonists, the effect is much more dramatic. There is a complete absence of a morphological neural plate: the only *Sox2* observed is in the rim of the blastopore. Dorsal mesoderm is also ablated and there is an expansion of posterior and lateral tissue. The authors argue that there is redundancy amongst the BMP antagonists and it is necessary to remove several before the real effect is observed of a lack of BMP inhibition. However, it does not explain why the application of BMP antagonists to the area opaca in chick embryos fails to elicit a neural induction response (Linker and Stern, 2004). Overall, these results suggest that neural induction is not a default but a response to a series of signals and events and that further factors are likely to be involved.

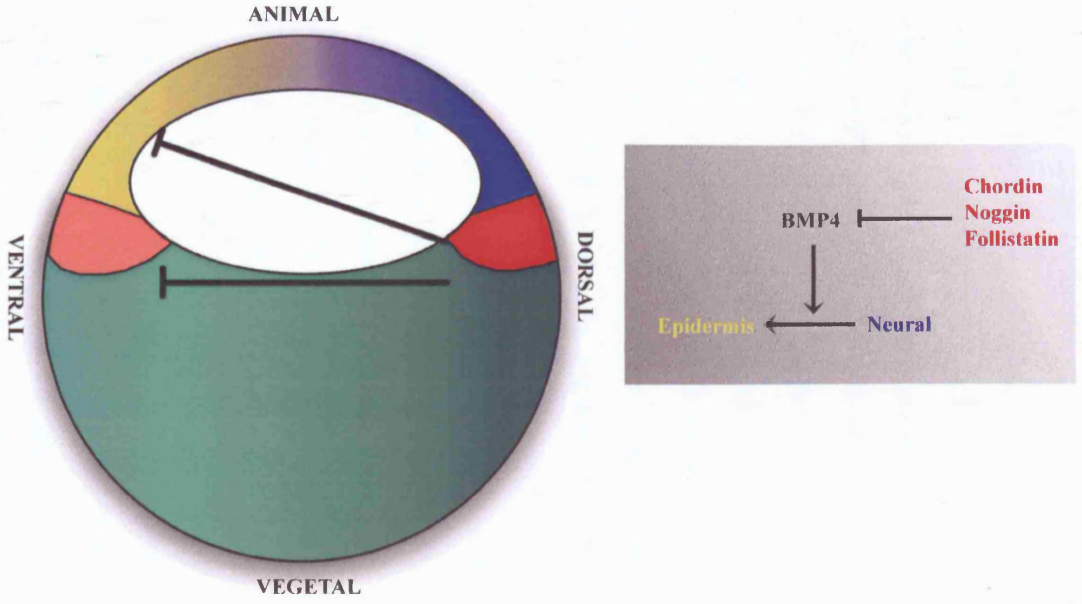


Figure 1.2 The 'Default Model' for neural induction. A gastrula stage *Xenopus* embyo is represented on the right. Cells will follow a neural fate (blue) unless they receive BMP signals that convert them to epidermis (yellow). 'Neuralisers' like Chordin, Noggin and Follistatin antagonise BMP signalling dorsally, allowing these cells to become neural. Modified from Stern, 2004.

1.4 Tissues and Signals Involved in Anterior-Posterior Patterning

1.4.1 Anterior specification - The Early Stages

i. The role of the extra-embryonic endoderm in anterior specification

The hypoblast forms as a sheet of extra-embryonic endoderm prior to the appearance of the primitive streak in the avian embryo and it was initially thought that grafts of the hypoblast could induce forebrain character (Eyal-Giladi and Wolk, 1970). Since this work on the hypoblast, it has been little investigated until recently. Over the last few years more and more evidence is accumulating to indicate that the extra-embryonic endoderm in chick and mouse plays a role in anterior specification (Thomas & Beddington, 1996; Varlet, Collignon & Robertson, 1997; Shawlot et al., 1999; Dufort et al., 1998; Acampora et al., 1998; Rhinn et al., 1998; Foley et al., 2000). When the AVE is removed from the mouse embryo during early gastrulation, expression of the forebrain marker *Hesx1* is reduced or absent from the anterior neurectoderm. The hindbrain marker, *Gbx2*, remains unaffected (Thomas & Beddington, 1996). Several mouse mutants with forebrain defects were reanalysed for defects in the AVE. Previously, Beddington & Robertson (1989) had discovered that if wild-type embryonic stem (ES) cells are injected into mutant blastocysts, they will contribute to embryonic lineages but rarely to the visceral endoderm. This assay was used to reassess the role of the AVE. The *Otx2* mutant has anterior brain deletions (Acampora et al., 1995). When wild-type ES cells are injected into mutant blastocysts, the phenotype is identical to those of the full mutant. However, when mutant ES cells are injected into wild-type blastocysts, the visceral endoderm can rescue the early anterior defects (Acampora et al., 1998; Rhinn et al., 1998). Similar results have been obtained for *Nodal*, *Hnf3B* and *Lim1* (Varlet, Collignon & Robertson, 1997; Shawlot et al., 1999; Dufort et al., 1998). These results give support to the notion that the AVE is required for forebrain formation.

The *Cripto* mutant is of particular interest in terms of AVE function. *Cripto* belongs to the EGF-CFC family of extra-cellular proteins which have been shown to be involved in several steps in early embryonic development in conjunction with the TGF-β protein, Nodal (Shen & Schier, 2000; Reissmann et al., 2001; Yan et al.,

2002; Minchiotti et al., 2002). In mouse, *Cripto* is expressed in the entire epiblast before gastrulation (Ding et al., 1998; Minchiotti et al., 2000). When the *Cripto* gene is knocked out, homozygous embryos die at 9dpc. The particularly interesting aspect of this mutant for A-P patterning is that it lacks a morphological primitive streak and node structures whilst retaining a functional AVE. The AVE develops normally but, instead of moving to the anterior side of the embryo, it remains at the distal tip. The resulting axis develops as concentric domains of expression of anterior neural markers, with the most anterior markers directly above the AVE and more posterior markers towards the extra-embryonic ectoderm (Liguori et al., 2003). Although this result could be interpreted as demonstrating the sufficiency of the AVE for head development, there are a few confounding factors. At 6-6.5dpc, the *Cripto* mutant has a molecular EGO (it expresses EGO markers: *FGF8*, *Lim1*, *T* and *goosecoid*) in the posterior epiblast (Ding et al., 1998) although it does not go on to develop a node. In mutants that still have a developed AVE but lack the EGO, such as *β-catenin* (Huelsken et al., 2000) and *Wnt3* (Liu et al., 1999), no neural tissue is formed. Even though the AVE in these mutants expresses AVE markers: *Cerr1* and *Lim1*, no early epiblast expression of *Otx2*, *Hesx1* or *En1* is ever observed (Huelsken et al., 2000; Liu et al., 1999). Furthermore, the mouse AVE is unable to induce anterior neural markers when grafted into non-neural ectoderm. In order to induce a fore/midbrain character, the AVE needs to be grafted together with a piece of anterior epiblast (prospective forebrain) for a weak induction and with both anterior epiblast and EGO (early gastrula organizer) for a strong induction of markers, *Otx2* and *En2* (Tam and Steiner, 1999).

Some have speculated that the AVE is a head organizer with distinct organizing abilities from the node and that this property is unique to mammals (Knoetgen et al., 1999a, 1999b, 2000). When a mouse node is grafted into the non-neural ectoderm, a secondary axis develops which lacks a morphological head (Beddington, 1994). There are several possible reasons to account for this. Firstly, the region in which the node is grafted might not be competent to respond to the signals that induce and pattern anterior neural tissue. Secondly, it is possible that the graft is too close to the prospective forebrain of the host and following the graft, the node only caudalises (i.e. generates caudal nervous system extending from a head shared by both axes. Thirdly, the node at this stage might have lost its ability to induce the most anterior markers and would be equivalent to the avian node after the prechordal region and

head process have emerged from the primitive streak (Dias and Schoenwolf, 1990; Storey, 1992; Foley et al., 1997); indeed, in mouse, the full streak stage includes embryos that already have a small head process primordium (equivalent to chick embryos at stages 4+/5). However, grafts of the early gastrula organiser (EGO) are still unable to induce forebrain character (Tam and Steiner, 1999). This might be because the EGO is too young and an intermediate-staged organizer might be required to pattern a whole axis (as also found in the chick; Storey et al., 1992). To test this, Kinder et al. (2001) used a region of anterior primitive streak from a mid-streak stage embryo, termed the MGO (mid-gastrula organizer) as a donor and grafted it into the epiblast of a host embryo. The host tissue is distinguishable from the graft tissue because the graft was derived from a mouse line expressing an *EGFP* transgene. The MGO produces a secondary axis that contains *EGFP*-expressing (graft-derived) cells. However, the overlying host ectoderm is thickened and expresses the neural marker *Sox2* and the anterior brain marker, *Otx2*. The reason suggested by the authors for the abilities of the MGO to induce forebrain when grafted is that it contains precursors for the anterior mesendoderm. Whilst the EGO contains a few of these precursors, the MGO incorporates the EGO and further anterior mesendodermal precursors (Kinder et al., 2001). This is analogous to the avian node at full inducing ability that contains within it the precursors for the head mesendoderm. Therefore, the MGO would appear to be the equivalent of the avian node from a stage 3-4 embryo (Storey et al., 1992) but there remains the possibility that the MGO is recruiting host cells to form part of the secondary neuraxis and it is not a true induction.

The rabbit embryo is strikingly similar in appearance to the avian embryo, especially at pre-streak and gastrulation stages. It is a useful link between mammals and birds because, whilst it is a mammal, it has a flat embryo like the chick and does not develop in the typical cylindrical shape of the mouse. If there is a distinct mechanism used by mammals for head induction and patterning it should be conserved between rabbit and mouse. If, however, the murine AVE evolved to compensate for the shape change of the embryo there will be similarities between the rabbit and the chick. Despite the fact that the full streak-stage mouse node fails to give rise to a secondary axis expressing anterior neural markers when grafted in the mouse (Beddington, 1994), when it is grafted into the border between area opaca and area pellucida in the chick embryo both it and the rabbit node can induce a secondary axis expressing all

the markers of rostro-caudal identity (Knoetgen et al., 2000). The caveat is that the site of the graft is critical when distinguishing between neural induction and regional specification. These node grafts appear to be placed at the border between the area pellucida and area opaca of the chick host. This region might receive some signals from the developing host embryo, and the graft might also recruit host cells to be incorporated into the ectopic structure. This might influence the result by generating a secondary axis that has not been induced by a graft that would not be capable of such an induction in the area opaca.

When the rabbit hypoblast is grafted into the margin between the area pellucida and the area opaca of a chick embryo at full streak stage, *Ganf*, a forebrain marker, is induced as well as *Sox3*. The ectoderm overlying the graft is thickened (Knoetgen et al., 1999). These grafts were left for 6-10 hrs before fixation. The authors tested the ability of the avian hypoblast or node to induce *Ganf* and cultured embryos for 6-18 hours. The chick hypoblast failed to induce the anterior neural marker whereas the node did. These data could reveal a difference between the chick and rabbit hypoblasts' role in induction and patterning. However, a couple of factors could cast some doubt on this interpretation. Firstly, the difference in the incubation time for the chick and rabbit graft might be critical and secondly, the mouse AVE, and if this is conserved across mammals, potentially the rabbit hypoblast also express *Ganf* whereas at the stages used, the avian hypoblast does not (Foley et al., 2000). This could reflect an earlier aspect in the role of *Ganf* in mammals compared to birds. Therefore, the evidence to suggest that the mammalian hypoblast, but not the avian hypoblast, is a distinct head organizer is not conclusive.

The avian hypoblast is involved in many important processes before and after gastrulation (Waddington, 1930; 1932; 1933; Foley et al., 2000; Bertocchini and Stern, 2002). It acts as an inhibitor of primitive streak formation, and its displacement away from the site of primitive streak initiation by the endoblast appears to be the trigger for streak formation (Bertocchini and Stern, 2002). This has been suggested to be the result of the hypoblast's ability to inhibit Nodal signalling. Nodal is required for the initiation of primitive streak development. The hypoblast expresses the Nodal antagonist, *cerberus*, which is not expressed by the endoblast. The displacement of the hypoblast by the endoblast was proposed to act by removing Nodal inhibition thus allowing streak formation to begin (Bertocchini & Stern,

2002). Consistent results have also been obtained in mouse: mouse mutants lacking both *Lefty-1* and *Cerberus* (both of which are Nodal antagonists expressed in the AVE) results in embryos with multiple primitive streaks (Perea-Gomez et al., 2002). Simultaneously with this role of the hypoblast in regulating primitive streak initiation, the hypoblast also contributes to direct Polonaise cell movements in the epiblast and thus the movement of prospective forebrain cells. In hypoblast rotation experiments, the anterior streak and anterior territories (along with primitive streak elongation) are reoriented following the direction of the displaced hypoblast (Waddington, 1930; 1932; Foley et al., 2000). This shows that the hypoblast can direct the movement of the prospective forebrain region; this movement might ensure that prospective forebrain cells remain away from the node, a potential source of caudalising signals that would otherwise interfere with this region acquiring a forebrain fate. A similar role has been proposed for the mouse AVE, which has be shown to undergo active migration in concert with the overlying epiblast (Srinivas et al., 2004); in addition to active migration, movement of the AVE also appears to be driven by cell proliferation in the posterior VE (Yamamoto et al., 2004).

The chick hypoblast shares expression profiles of many genes with the mouse AVE [Table 1]. A few differences to be noted are that *Hesx1/Rpx/Ganf* is not expressed in the hypoblast until stage 4+ when it is also expressed in the prechordal mesoderm and overlying epiblast (Foley et al., 2000). *Hex* expression in chick is not as spatially restricted as in the mouse prior to gastrulation (but perhaps the mouse AVE is somewhat smaller than the chick hypoblast). *Nodal* and its antagonist *Lefty1* do not appear to be expressed in the avian hypoblast although there might be other members of these families which have not yet been identified (Bertocchini et al., 2002). Conversely, *crescent*, a Wnt antagonist, is expressed in the hypoblast but has not yet been identified in mouse (Pfeffer et al., 1997; Foley et al., 2000; Chapman et al., 2002).

A role for the hypoblast in neural induction and anterior specification was investigated by grafting it into the inner third of the anterior area opaca at stage 3+/4 (Foley et al., 2000; Streit et al., 2000). The hypoblast could induce the expression of *Otx2* in the area opaca after 4 hours and that of *Sox3* after 8 hours. However, expression of both of these markers disappeared after 18 hours in culture and the hypoblast never induced definitive neural marker, *Sox2* (Foley et al., 2000). *ERNI* was also induced by the hypoblast after 3 hours (Streit et al., 2000). This is

reminiscent of a full streak stage-node which, when grafted in the area opaca and removed after 5 hours, induces expression of *Sox3* and *ERNI* which is then lost when the host embryo is cultured for longer (Streit et al., 2000). The ability of the hypoblast to induce markers transiently is also similar to the AVE in the mouse. The AVE is required for the expression of *Otx2* in the epiblast but subsequent mesodermal interactions are required to maintain its expression in the anterior neural plate (Ang et al., 1994; Acampora et al., 1998; Rhinn et al., 1998; Shawlot et al., 1999). Therefore, whilst the hypoblast/AVE appears to be involved in the earliest stages of anterior specification, it does not possess the ability for either full neural induction or for anteriorisation of more caudal neural tissue.

ii. Fish and Frog Equivalents of the AVE/Hypoblast

There is no definitive homologous structure in zebrafish or *Xenopus* to the hypoblast or AVE. In teleost fish an extra-embryonic tissue known as the yolk syncytial layer (YSL)(Kimmel et al., 1995) is formed from the marginal blastomeres. It forms a narrow ring around the blastodisc edge (Kimmel at al., 1985) before rapidly spreading beneath the blastodisc. At the dome stage it forms a complete syncytium that remains throughout embryogenesis. As early as 1936, Oppenheimer proposed that this yolk cell layer provides signals necessary for embryo formation in the teleosts *Fundulus* and *Perca*. The YSL has been suggested to be the teleost Nieuwkoop centre (reviewed in Schier and Talbot, 1998; Solnica-Krezel, 1999; Mizuno et al., 1996), a region described in *Xenopus* that is established in the vegetal hemisphere in response to maternal patterning events and which functions as an inducer of Spemannn's Organizer (Gerhart et al., 1989).

In the rainbow trout, *Salmo gairdneri*, a bilaterally symmetrical blastodisc was removed from the underlying YSL and replaced with a younger, radially symmetrical blastodisc. The new blastodisc became bilaterally symmetrical in a way that matched the YSL suggesting that the YSL is able to pattern and direct cell movements of the overlying ectoderm (Long, 1983). The movements of the YSL were studied further using vitally stained YSL nuclei in axiating zebrafish embryos and imaging them using 4D confocal microscopy. It was found that the pattern of movement of the YSL corresponds to that in the overlying blastoderm (D'Amico and Cooper, 2001). This is reminiscent of the finding that the hypoblast directs the movement of epiblast

cells (Waddington 1930; 1932; 1933; Foley et al., 2000). Recently, several genes have been found to be expressed in the YSL, some of which are also expressed in the AVE and hypoblast; examples are *Hex* (Ho et al., 1999) and *Blimp1* (Chang et al., 2000). At the beginning of gastrulation, *Hex* is located in the dorsal half of the YSL where it remains until the end of gastrulation; this is a region that underlies the prospective neural plate (Schneider et al., 1996; Ho et al., 1999). Experiments by Ho et al. (1999) revealed that *Hex* expression is initially regulated by the maternal Wnt pathway and subsequently by BMP. Furthermore, *Hex* is capable of down-regulating *BMP2b* and *Wnt8* as well as expanding the domain of *chordin* expression. *Bozozok*, also expressed in the YSL, is thought to promote anterior neurectodermal fate through antagonism of BMP and Wnt signalling, supporting the idea that the YSL has a role in BMP and Wnt inhibition (Fekany-Lee et al., 2000; Fekany et al., 1999). *Blimp1/Prdm1*, which is also expressed in the mouse AVE (Chang et al., 2000), has been detected in the YSL and is proposed to limit the function of the organizer by restricting the domain of *chordin* expression (Wilm and Solnica-Krezel, 2005). These data suggest that the YSL has similar properties to the AVE and hypoblast: it lies beneath the prospective neural plate where it moves in concert with, and might direct, blastoderm cell movements; it expresses *Hex* and *Blimp1*, genes also expressed in the AVE, the former shown to be required for head formation in the mouse (Martinez Barbera et al., 2000; Chang et al., 2002); it has a potential role in inhibiting BMP and Wnt signalling.

The *Xenopus* anterior embryonic endoderm and mouse AVE express homologous genes (Beddington and Robertson, 1998) that include *Hex* (Newman et al., 1997; Thomas et al., 1998), *Xblimp1* (de Souza et al., 1999), *Cerberus* (Bouwmeester et al., 1996; Belo et al., 1997; Biben et al., 1998) and *Dkk1* (Glinka et al., 1998; Osada & Wright, 1999; Schneider & Mercola, 1999). However, ablation of this region does not affect forebrain development (Schneider & Mercola, 1999). In *Xenopus*, the anterior endoderm is definitive, giving rise to the liver (Bouwmeester et al., 1996) and foregut, and when compared to the mouse definitive endoderm, these genes are still homologous. *Hex* and *cerberus-like* are expressed in the mouse definitive endoderm that will give rise to the liver and (Belo et al., 1997; Biben et al., 1998; Shawlot et al., 1998; Thomas et al., 1998). Therefore, the Xenopus anterior endoderm is partly similar to the AVE but also partly similar to the mouse anterior

definitive endoderm. However, similar movements have been discovered in the Xenopus anterior endoderm and the chick hypoblast (Arendt and Nübler-Jung, 1999) and these were further described as extensive rotational movements, which were termed "vegetal rotation" (Winklbauer and Schürfeld, 1999). When anterior endoderm is grafted into the blastocoele of a host embryo it has weak head inducing activity (Einsteck experiment; Mangold, 1933; Bouwmeester et al. 1996) however when this endoderm is removed from young gastrula stage embryos the head develops normally unless the prechordal plate is also removed (Schneider and Mercola, 1999), suggesting that the anterior endoderm is not required for head development and that the prechordal plate is more important. These data are inconclusive regarding the homology of the Xenopus anterior endoderm to the mouse extraembryonic endoderm.

iii. Factors involved in the activity of the hypoblast

Several factors have been proposed as signals involved in the ability of the hypoblast to induce transient expression of pre-neural markers in the area opaca (Foley et al., 2000; Streit et al., 2000) including RA, FGF, Wnt-, Nodal- and BMP-antagonism (Bertocchini and Stern, 2002; Halilagic, Zile & Studer, 2003; Knezevic & Mackem, 2001; Knezevic, Ranson & Mackem, 1995; Streit et al., 2000; Wilson et al., 2000). FGF8 can induce *Sox3* and *ERNI* when grafted in the area opaca and it is itself expressed at low levels in the hypoblast at pre-streak stages (Streit et al., 2000). Wilson et al. (2000) reported that *FGF3* mRNA can be detected by RT-PCR in explants of stage XII embryos along with its receptor, *FGFR2b*. However, it is unclear whether these explants included the lower layer. These authors also reported that FGF signalling is required for the epiblast to express *Sox3* and *FGF3* after being cultured for 40 hours as an explant. This might be an indirect effect of removing BMP inhibition because when both FGF and BMP are blocked, the expression of *Sox3* is maintained (Wilson et al., 2000). FGF is also detectable at the earliest stages in mouse. *FGF3/int-2* (Wilkinson et al., 1988) and *FGF8* (Crossley and Martin, 1995) are expressed in the visceral endoderm and although neither of the single mutants has a defect related to AVE formation (Mansour et al., 1994; Sun et al., 1999) there might be some overlap in function.

RA has also been suggested to play a role in anterior specification. The RA synthesis enzyme, *RALDH2*, is expressed by the hypoblast at stage 4 (Halilagic et al., 2003). *cNot1* and *cNot2* are expressed in the epiblast prior to streak formation. At pre-streak stages it has been shown that, when cultured without the hypoblast, *cNot1* and *cNot2* fail to be induced in the epiblast (Knezevic et al., 1995; Knezevic & Mackem, 2001). Knezevic & Mackem (2001) went on to show that RA is able to induce *cNot1* and *cNot2*, an effect enhanced by FGF4, suggesting that it is RA, potentially produced in the pre-streak stage hypoblast, which is responsible for activating these genes. Furthermore, RA, when locally applied, affects the direction of the primitive streak orientation reminiscent of the hypoblast rotation experiments (Knezevic & Mackem, 2001). However, when RA is applied, the streak and prospective forebrain rotate away from the source whereas the streak and forebrain reorient in the same direction as a rotated hypoblast (Waddington, 1930; Waddington, 1932; 1933; Foley et al., 2000). This suggests that RA might be produced by the hypoblast but it is not necessarily active throughout the layer. RA signalling is also required for head development in *Xenopus* embryos and morpholinos against the RA receptor *RARα* produce anterior truncations when injected (Shiotsugu et al., 2004). Therefore RA is a candidate signalling molecule for early anterior specification.

Wnt and BMP antagonists are expressed in the hypoblast (Foley et al., 2000; Chapman et al., 2002) and it is known that there are Wnts and BMPs expressed in the area opaca of pre-streak embryos but not in the area pellucida (Skromne and Stern, 2001; Chapman et al., 2002). Therefore, one role of the hypoblast might be to down-regulate these factors in order to induce the characteristic epiblast markers and anterior neural character. Hence, whilst there is indirect evidence for the roles of FGFs, RA, Wnt- and BMP-inhibition in hypoblast-mediated induction of pre-neural, pre-forebrain markers, there have been no definitive experiments to examine this possibility.

Table 1 Summary of genes expressed in the mouse AVE and chick hypoblast

Gene Expressed	Role	K.O.Phenotype	Mouse AVE	Reference	Chick Hypoblast
Otx2	Homeobox	Early-development lethal. Embryos fail to specify rostral neurectoderm and do not gastrulate properly	Yes	1,2	yes
Goosecoid	Homeobox	Normal and fertile	Yes - restricted during gastrulation	3,4	Yes
HNF3B	Transcription factor	Affects primitive streak, node and notochord formation	Yes - restricted during gastrulation	5,6,7	Yes
Cerberus	Secreted BMP, Nodal and Wnt antagonist	No phenotype	Yes - restricted during gastrulation	8,9,10	Yes
Hesx1/Rpx/Ganf	Homeobox	Variable rostral CNS defects	Expression starts during gastrulation	11,12,13,14	Yes
Nodal	TGF-like transcription factor	Development arrested at early gastrulation: contains little or no embryonic mesoderm	Yes	15,16,	Yes
Hex	Homeobox	Variable rostral forebrain truncations	Yes - restricted to AVE from 5.5 dpc	17,18	Yes
Lim1	Transcription factor	Lacks rostral head structures	Yes	20,21,	Yes
Dkk1	Secreted Wnt antagonist	Rostral head truncations at the level of the midbrain/hindbrain boundary. AVE initially expressed correct markers but fails to develop	Yes	21,22,23	Yes
Crescent	Frizzled-like secreted factor: Wnt antagonist		No mouse homologue	24,	Yes

References: 1. (Acampora et al., 1995), 2. (Simeone et al., 1995), 3. (Wakamiya et al., 1998), 4. (Blum et al., 1992), 5. (Ang et al., 1994), 6. (Dufort et al., 1998), 7. (Sasaki & Hogan, 1993), 8. (Shawlot et al., 2000), 9.(Belo et al., 1997), 10. (Shawlot, Deng & Behringer, 1998), 11. (Dattani et al., 1998), 12, (Martinez Barbera et al., 2000), 13. (Thomas & Beddington, 1996), 14. (Hermesz, Mackem & Mahon, 1996), 15.(Conlon et al., 1994), 16. (Varlet et al., 1997), 17. (Martinez Barbera et al., 2000), 18. (Thomas, Brown & Beddington, 1998), 19. (Shawlot et al., 1999), 20. (Shawlot & Behringer, 1995), 21. (Mukhopadhyay et al., 2001), 22. (Glinka et al., 1998), 23.(Pearce, Penny & Rossant, 1999), 24. (Pfeffer, De Robertis & Izpisua-Belmonte, 1997).

1.4.2 Anterior Specification - The Later Stages

The early extra-embryonic regions of the mouse, chick and zebrafish have been shown to be involved in the early stages of anterior specification and, in the case of the mouse AVE, shown to be required for head formation, as discussed above. During gastrulation, extra-embryonic endoderm is displaced anteriorly (Vakaet, 1970; Stern and Ireland,1981; Stern, 1990; Thomas and Beddington, 1996; Srinivas et al., 2004; Yamamoto et al., 2004) and other tissues come to underlie the prospective forebrain and are important in its development. These, along with factors involved in their activity, will be reviewed below.

i. The Node can induce rostro-caudal neural markers

The node can induce and pattern a complete secondary axis and it was shown that the node is competent to induce a complete axis including head structures when taken from stage 2-4 embryos (Dias and Schoenwolf, 1990; Storey et al., 1992). However, after stage 4 the node is unable to induce anterior markers and subsequently rapidly loses the ability to induce neural tissue at all but can still self-differentiate and express posterior neural markers (Dias and Schoenwolf, 1990; Storey et al., 1992). This indicates that the properties of induction and posterior regional specification are separable. It has been proposed that the reason the node loses its ability to induce anterior neural structures after stage 4 is because this is when the prechordal mesendoderm (PME) and head process emerge and within this population of cells reside signals for anterior neural induction (Dias and Schoenwolf, 1990; Storey et al., 1992; Foley et al., 1997; Schneider and Mercola, 1999; Saúde et al., 2000; Kinder et al., 2001).

ii. The head process can induce anterior neural tissue

The precursors of the head mesoderm reside within the pool of cells that emerge from the node at stage 4+. These resolve into the PME that proceeds ahead of the cephalic chordamesoderm: the head process. When node regression commences at stage 6, the head process starts to become elongated caudally by addition of cells to form the notochord proper (the portion of the chordamesoderm from the caudal hindbrain to the caudal tip of the spinal cord). In the chick, when rostral and caudal head process (RHP and CHP, corresponding to prosencephalic and

metencephalic/rhombencephalic levels of the head process respectively) are grafted into the area opaca, it was found that RHP often induces a neural-tube type structure which expresses the markers *En2*, *FGF8* and *Krox20* consistent with a midbrain/anterior hindbrain character (Rowan et al., 1999). The CHP induces neural structures occasionally but they express the caudal hindbrain/anterior spinal cord markers, *Hoxb4* and *Sax1*. This shows that, within the head process, there is specific neural regional inducing ability that is distinct between the rostral and caudal part. The assay above tested induction ability of the head process. To look at its patterning properties, isolated explants were taken from the rostral neural plate, which express *tailless, Otx2, En2* and *Krox20* (both prosencephalic and hindbrain markers) when cultured in the area opaca alone. When explants from this region are combined with RHP, *En2* and *Krox20* are not expressed in the RNP. This shows that the RHP can restrict the expression of markers; mes/metencephalic and hindbrain markers are no longer present but more rostral markers are. Interestingly, although the CHP can reduce the expression of *Otx2* in rostral neural plate explants, it is not able to induce more posterior markers (Rowan et al., 1999). Therefore, the patterning ability of the head process would appear to be to refine and restrict the range of markers that the RNP is capable of expressing.

These two sets of experiments argue that there are signals in the head process that can induce neural tissue, and ascribe it a specific A-P character depending on the level of head process grafted; and it can confer regional character to induced neural plate. In the CHP, there are signals that can restrict anterior neural markers without inducing more posterior ones, suggesting that anterior marker restriction and posterior marker induction might be separable.

Experimental evidence from the mouse suggests that anterior specification and posterior specification are distinct. Ang et al. (1994) suggested two possible hypotheses for continued *Otx2* expression in the anterior neurectoderm and restriction from the entire epiblast early in development to the anterior domain after gastrulation. Potentially a positive signal is required to maintain *Otx2* and only the anterior ectoderm is exposed to this signal, emanating from the underlying mesoderm. Alternatively, a repressive signal might be required to prevent *Otx2* expression in the posterior epiblast. *Otx2* is only stable in neurectoderm explants after early-streak stage. In culture, explants taken from younger embryos grown with anterior mesendoderm retain *Otx2* expression but those grown with posterior

mesendoderm only maintain expression in 9% of cases, which is lower than for ectoderm cultured alone. If explants of ectoderm are cultured until they switch off *Otx2* and then combined with anterior mesendoderm, *Otx2* is switched back on and also, posterolateral ectoderm, which at this time does not express *Otx2*, can be induced to express it when cultured with anterior mesendoderm. Posterior mesendoderm explants can reduce or abolish *Otx2* expression in epiblast explants from late streak embryos suggesting that the former emits a repressive signal. RA treatment at early streak stages causes a premature downregulation of *Otx2* expression. It is interesting that even though the RA is produced globally, there is still a gradient of expression of *Otx2*, higher anteriorly. Perhaps only the posterior expression is responsive to RA or, since there are separate *Otx2* enhancers for early epiblast and for anterior neural expression (Kurokawa et al., 2004), only the former may be responsive to different levels of RA. These results, in combination, are very interesting. They show that there is an early expression of *Otx2* in the epiblast (requiring signals from the AVE: Acampora et al., 1998; Rhinn et al., 1998) that requires further signals from the anterior mesendoderm, and which require *Otx2* expression in this tissue, to be maintained (Ang et al., 1994; Rhinn et al., 1998). The posterior mesendoderm also emits a repressive signal that downregulates *Otx2* in the posterior epiblast. These findings suggest that early anterior markers induced by extra-embryonic structures must be maintained in the anterior neurectoderm by instructive signals from anterior mesendoderm. Furthermore, there is not necessarily just a progressive caudalisation of the neuraxis but rather anterior markers must also be actively repressed in the posterior neural plate.

iii. The Prechordal Mesendoderm (PME) can anteriorise neurectoderm

Once the PME separates from the chordmesendoderm the two can be distinguished by their expression pattern. The expression of *chordin* is lost from the PME and retained in the head process, *goosecoid* is retained in the PME but lost in the head process and *BMP7* is induced in the PME (Dale et al., 1999; Vesque et al., 2000). The anterior head process can induce neural tissue having a specific A-P character when grafted into the area opaca (Rowan et al., 1999). To investigate whether the avian PME has similar properties it was grafted from a stage 5 embryo into the area opaca but it could not induce neural markers (Foley et al., 1997). The node loses its ability to induce anterior neural markers at a stage when the PME has emerged (Dias

and Schoenwolf, 1990; Storey et al., 1992). It is possible that it is the PME cells in the node that have the forebrain inducing capacity. To test this, a PME graft and a node graft, both taken from stage 5 donors, were grafted together into the area opaca. In this instance, a full anterior-posterior axis was generated indicating that the PME could indeed rescue the ability of the node to induce forebrain markers. The PME can also induce ectopic anterior neural markers (Foley et al.1997; Pera and Kessel, 1997), *tailless* and *Otx2*, but not hindbrain marker, *Krox20* when grafted in the area pellucida, which was not the result of recruiting induced cells from the host axis. Furthermore, it was shown that if a PME graft is placed next to the prospective hindbrain of a stage 4 donor, the fate of the hindbrain cells is altered and they start to express the anterior neural marker, *tailless* (Foley et al., 1997). However, it is unclear whether this is through active anteriorisation or through the repression of caudalising signals. In summary, in the chick the PME is not capable to induce neural markers like the head process but it can induce forebrain markers in the area pellucida and it can anteriorise prospective hindbrain to adopt a forebrain character.

In contrast, the PME has been shown to have head inducing abilities in *Xenopus* (Mangold, 1933). More recently, experiments have been performed to analyse the inducing properties of different parts of the organizer. The Einsteck procedure, whereby grafts are inserted in the blastocoele cavity of a blastula or early gastrula host, or explant cultures of organizer tissue and responsive ectoderm, revealed that the prospective PME cells in the vegetal portion of the organizer are able, on their own, to induce a head (Zoltewicz and Gerhart, 1997). Following these experiments, Schneider and Mercola (1999) analysed the PME for expression of markers associated with the mouse AVE. They found *Xanf* as well as the previously reported *Xhex1* *XOtx2* and *XLim1* (Newman et al., 1997; Zaraisky et al., 1995; Sasai et al., 1994; Pannese et al., 1995) to be co-expressed in the PME region of the organizer. When this region was removed, head development was impaired to varying degrees (Schneider and Mercola, 1999). Therefore, the PME is required for normal head development in Xenopus. Also, it is necessary to inhibit BMP and Wnt signalling for normal head development (Glinka et al., 1997) and both Wnt and BMP antagonists are expressed in the *Xenopus* PME supporting the notion that this tissue is involved in head formation (Wnt antagonist *Dkk1*, Glinka et al., 1998; reviewed by Niehrs, (1999) and BMP antagonist *noggin*, Hongo et al., 1999).

These data suggest that the PME is able to induce anterior neural identity in the embryo but only in tissue that has received some prior signals from the organizer. In Xenopus and zebrafish, it has been suggested to have a more potent role as a neural inducer and head inducer (reviewed in Kiecker and Niehrs, 2001).

iv. Hypoblast/Anterior Definitive Endoderm (ADE) and anterior specification

At the full primitive streak stage, the early lower layer comprising hypoblast and endoblast has been displaced by the embryonic anterior definitive endoderm. The role of the anterior definitive endoderm (ADE) has been studied with regard to anterior specification in the chick (Knoetgen et al., 1999, Withington et al., 2001; Chapman et al., 2003). This role was analysed by removing the ADE at various stages of embryonic development (Withington et al., 2001). At stages prior to stage 5, the ADE regenerates but when removed at stage 5, it does not regenerate and there is a loss of proper forebrain and general head formation. Initially, correct expression domains are maintained: *Sox3*, *Otx2* and *Ganf* are normal. The first evidence of anterior defects is the loss of *FGF8* in the anterior neural ridge (ANR). It has been suggested that the expression of *FGF8* in the ANR in mouse is required for the induction or maintenance of *Bf1* (Shimamura and Rubenstein, 1997). Indeed, telencephalic expression of *Bf1*, which is normally expressed at the 7-8 somite stage, is absent or severely reduced in ADE-removal experiments as is the later expression of another forebrain marker, *Ganf*. Other anterior neural markers are not expressed in their correct domains but it is unclear as to whether this is because those structures are absent or if the markers are not being maintained. *Pax6* is normally expressed in the forebrain, in rhombomere 1 in the hindbrain and in the spinal cord at stage 12. However, the spinal cord boundary is shifted anteriorly to just caudal to the *Pax6* expression domain in rhombomere 1 after removal of the ADE. This could indicate either a loss of intermediate structures or a lack of repressive signals to inhibit caudalisation. The latter might be the case because if the ADE is taken from a stage 5 embryo and grafted beneath the prospective hindbrain, an expanded domain of *Ganf* expression results (Withington et al., 2001). This is a similar result to what is observed after grafting the PME next to the prospective hindbrain (Foley et al., 1997).

In the experiments performed by Withington et al. (2000) removal of the ADE did not initially affect the prospective forebrain expression of *Otx2* or *Ganf*. However,

when stage 3/3+ chick embryos are bisected (directly rostral to the node so that the rostral portion can be cultured alone without influences from the organizer) and cultured with or without the lower layer it was discovered that the endodermal layer is required for *Gsc* expression to be induced in the epiblast (Chapman et al., 2003). The discrepancy between these two studies could be explained by the fact that in the Withington et al. (2001) study used whole embryo cultures and therefore the epiblast might receive signals from tissues removed in the bisected embryos used by Chapman et al., 2003).

Withington et al., (2001) also showed that when the ADE is removed, the PME stops expressing *Hex1*. Vesque et al. (2000) suggested that signals from the anterior endoderm at stages 3+ and 4+, when it is either a mixture of hypoblast and ADE or just ADE, are required for PME cells to express distinct markers from the head process (at around stage 6-7) by down-regulating *chordin*, maintaining *goosecoid* and inducing *BMP7* in the PME. These effects can be mimicked by a combination of BMP2 and BMP7 (to induce *BMP7* and down-regulate *chordin*) and Activin to maintain *goosecoid*. Anterior endoderm can induce PME character in newly formed notochord from stage 10 embryos (Vesque et al., 2000). Therefore, the specification of the PME and its separation from the head process might be in part due to the hypoblast and anterior definitive endoderm. This could suggest an indirect role for the ADE in head formation whereby it is required for the correct specification of the PME that in turn influences the neurectoderm. It is interesting that the PME expresses *BMP7* and that it down-regulates *chordin*. It is generally thought that BMP signalling needs to be blocked for neural induction (see the review of Neural induction above). In this context, the observations that the PME expresses *BMP* whilst the head process expresses BMP antagonists (Dale et al., 1999; Vesque et al., 2000) does fit with the results that the head process can induce neural tissue in the area opaca whereas the PME cannot (Foley et al., 1997; Rowan et al., 1999) if BMP must be inhibited for cells to adopt a neural fate (Hemmati-Brivanlou & Melton, 1997).

1.4.3. Molecular Signals involved in Head Development

i. BMP antagonism is required for head formation

Chordin is the Xenopus homologue of the Drosophila gene, *short gastrulation* (*sog*). It is a relatively abundant secreted protein produced by organiser cells during

Xenopus gastrulation. It specifically binds to mature BMP in the subnanomolar range and acts extracellularly by preventing BMP from binding to its receptor (Piccolo et al., 1996). In *Xenopus* its expression is restricted to regions with known organiser properties. It is possible to induce *chordin* by microinjection of the organiser-associated mRNA encoding *goosecoid*, *Lim1* and *Not1* (Sasai et al., 1995; Sasai et al., 1994; Taira et al., 1994). Noggin, another BMP antagonist, is not induced by these proteins. Although both Noggin and Chordin can dorsalise at 1nM levels, 15.5nM of Noggin is required for neuralisation whereas 1nM of Chordin is able to neuralise (Piccolo et al., 1996). This suggests that Chordin is a stronger nerualiser than Noggin.

The mouse mutant for *chordin* develops relatively normally at early stages (Bachiller et al., 2000). However, it was thought that its activity might be compensated by Noggin, which overlaps in expression with *chordin* at mid-gastrulation (McMahon et al., 1998). *Noggin* mutants also undergo normal gastrulation and anterior patterning at early stages (Bachiller et al., 2000). Double mutants have much more severe and early phenotypes. Looking at 8.5 days post-coitum (dpc), embryos express virtually no *Six3* (an anterior neural marker). The AVE forms normally and expresses the usual markers like *cerberus-like* and *Hesx1* but by 7.5dpc these markers have disappeared (Bachiller et al., 2000). This suggests that initial correct expression of AVE markers is not sufficient to maintain an anterior neural state and that further inhibition of BMP by Chordin and Noggin is required for head development. In the chick, *chordin* starts to be expressed at stage XIII posteriorly, in Koller's sickle cells (Streit et al., 1998). It is later expressed in the primitive streak, by stage 3 being confined to the anterior portion of the streak and the forming node. Subsequent expression is restricted to the notochord and the node. When a node from a full-streak stage embryo is grafted into the area opaca of a host at the same stage for 3-5 hours and then removed, the expression of *Sox3* induced during this time is only transient. If *chordin* expressing cells are added after removing the node graft, *Sox3* is maintained. However, the addition of chordin is not sufficient to induce *Sox2* expression in the same experiment (Streit et al., 1998) even in combination with FGF and/or other BMP- and/or Wnt-antagonists (Sheng et al., 2003; Linker et al., 2004). This shows that pre-neural genes can be induced only transiently unless they receive a further signal to stabilise their expression. In the chick it seems that the inhibition

of BMP by Chordin can maintain this expression but it is not sufficient to induce definitive neural tissue.

In fish, *chordin* mutants have been isolated and named *chordino* (or *din*), showing a mild ventralisation but normal A-P patterning (Schulte-Merker et al. 1997). However, it was postulated that the lack of A-P phenotype is due to some compensation by other BMP antagonists (shown to be true in the mouse by Bachiller et al., 2000 and in Xenopus by Khokha et al., 2005 and also reviewed above) and so double mutants for *chordin* and *bozozok* were created (Gonzalez et al., 2000). The phenotypes of these double mutants exhibited two levels of severity, the lesser being a lack of notochord, anterior truncations, multiple fin folds and an accumulation of blood near the anus, and the more severe phenotype also included a complete lack of head and trunk structures but a tail developed with 10 somites. To test whether this stronger phenotype in the double mutants is a result of further decreasing the inhibition of BMP the expression of *BMP4* was analysed. The ventrolateral domain of *BMP4* is expanded dorsally during gastrulation (6.5 hours post fertilization, or hpf) in the double mutants much more than in either of the single mutants. Since head structures are absent in the double mutants and, concomitantly, the forebrain marker *Six3*, the mid/hindbrain marker *pax2.1* and the hindbrain marker *krox20* are all missing, the possibility that cells with a neural fate had been transformed into epidermal cells was investigated. *gata3*, a marker of prospective non-neural ectoderm, was expanded slightly in the single mutants but in the double mutants it spread dorsally, revealing a dramatic decrease in neural ectoderm territory (all from Gonzalez et al., 2000). In summary, BMP must be repressed in dorsal/anterior regions of the embryo for normal head development. There are multiple BMP antagonists and, in loss of function experiments, often more than one of these antagonists must be knocked down for the effects on head development to be fully revealed.

ii. Wnt Antagonism is required for anterior specification

a. Introduction

Another pathway that needs to be blocked for head development is the Wnt pathway (reviewed in Yamaguchi, 2001). An example of this requirement is found in the zebrafish *Tcf3* mutant, *headless*, which lacks eyes, forebrain and part of the

midbrain. The phenotype of this mutant is the result of a derepression of Wnt target genes in anterior domains that are normally repressed by TCF3. *Tcf3* expression, and Wnt antagonism, is therefore critical for anterior neurectoderm patterning (Kim et al., 2000). Another Wnt antagonist, *Tlc*, has been shown to be crucial in telencephalic development in zebrafish (Houart et al., 2002). The anterior neural border (ANB) cells are able to induce telencephalon markers when grafted into more posterior positions of the ectoderm (Houart et al., 1998). This ability is mimicked by *tlc,* which is secreted by the ANB cells. When *tlc* is repressed, the telencephalon fails to develop correctly. It is thought that *tlc* might regulate the size of the forebrain territory by regulating the level of Wnt signalling and by inhibiting its activity, allowing normal forebrain development (Houart et al., 2002). Graded Wnt signalling can also caudalise the anterior neurectoderm in *Xenopus* and chick (Kiecker and Niehrs, 2001; Nordstrom et al., 2002).

Secreted Wnt proteins signal through membrane-bound frizzled (frz) receptors. Canonical Wnt signalling, through the β-catenin pathway, is transduced by two receptor families. Frizzled proteins and lipoprotein-receptor-related proteins 5 and 6 (LRP5/6) bind Wnts and transmit their signal by stabilizing intracellular β-catenin. Wnt/β-catenin signalling is inhibited by the secreted protein Dickkopf1 (Dkk1), a member of a multi- gene family, which induces head formation when injected into amphibian embryos. Dkk1 has been shown to inhibit Wnt signalling by binding to and antagonizing LRP5/6 (reviewed in Jones and Jomary, 2002). The transmembrane proteins Kremen1 and Kremen2 are high-affinity Dkk1 receptors that functionally cooperate with Dkk1 to block Wnt/-catenin signalling (Mao et al., 2002).
Whilst Dkk1 can supposedly antagonise the canonical Wnt pathway alone, upstream regulators, secreted frizzled related proteins, which are secreted glycoproteins like Wnt but structurally resembling the frz receptors, inhibit both canonical and non canonical pathways by binding to Wnt or the frz receptor (reviewed in Jones and Jomary, 2002). Crescent is an example of a secreted frizzled related protein (Pfeffer et al., 1997; Pera and De Robertis, 2000). The canonical Wnt pathway results in transcriptional activation whereas the non-canonical pathway has been suggested to be involved in reorganisation of the cytoskeleton and morphogenetic movements for example in convergent extension (Heisenberg et al., 2000; Tada et al., 2002; Jones and Jomary, 2002).

b. Crescent and anterior neural specification

In *Xenopus*, *crescent* is expressed on the dorsal side of the blastopore and later in the prechordal plate (Pera and De Robertis, 2000; Bradley et al., 2000) and in the chick it is located in the hypoblast at pre-streak stages and later in the anterior definitive endoderm and it subsequently becomes restricted to the prechordal endoderm (Pfeffer et al., 1997; Chapman et al., 2002; Chapman et al., 2004).

Crescent has been shown to be a direct inhibitor of Wnt8c in chick (Marvin et al., 2001). The effects of Crescent have also been tested in Xenopus. *Crescent* (but not *frzb1*) causes cyclopia when overexpressed. This is not due to a loss of anterior brain as cyclopia might suggest because, when analysed with molecular markers, it was found that the *Otx2*-expressing domain is expanded with a loss of the *Krox20* region (Pera & De Robertis, 2000). *Crescent*-targeted morpholinos reduce expression of anterior markers revealing that Crescent acts to anteriorise the embryo. This might be mediated by inhibiting convergent extension (Shibata et la., 2005: in press). When ectopically expressed, *crescent* prevents the embryo from undergoing convergent extension (CE) without inhibiting tissue specification and therefore could normally prevent these movements in the head region, which does not undergo CE (Shibata et la., 2005: in press).

c. Dkk1 and anterior specification

In the chick, *dkk1* is expressed in the hypoblast and later in the anterior definitive endoderm and the node and subsequently in the notochord (Foley et al., 2000; Bertocchini et al., 2002; Chapman et al., 2002; Chapman et al., 2004 and personal observations). Dkk1 has very strong head inducing activity when injected into *Xenopus* embryos (Glinka et al., 1998) and is required for head formation in mouse (Mukhopadhyay et al., 2001). In *Xenopus* general overexpression of *dkk1* at blastula stage results in an enlarged head and short trunk. Dkk1 synergises strongly with *tbr* (a dominant negative construct of a BMP2/4 receptor) to induce organiser markers such as *gsc*, *Otx2* and *chordin* and this combined Wnt- and BMP-inhibition results in a complete secondary axis including an ectopic head with two eyes. This is more powerful than frzb1, which can only produce one eye in the same assay. When anti-DKK1 antibodies are injected into the blastocoele, embryos develop with

mircocephaly in 100% of cases. These embryos had reduced or absent cement glands and were cyclopic at tadpole stage with 5% lacking head structures all together. Upon sectioning, it was discovered that the remaining axis developed normally and only the neurectoderm and mesoderm of the head were affected (Glinka et al., 1998). The Wnt antagonist *frzb*, combined with *tbr*, (Glinka et al., 1997) and *cerberus* (Bouwmeester et al., 1996), when injected into *Xenopus* cause the formation of a secondary axis that is cyclopic. DKK1 is so much more efficient at inducing a second head when injected into *Xenopus*, with two eyes (Glinka et al.,1998) because its overexpression results in the formation of a proper secondary PME that extends ahead of the neurectodermal *goosecoid* expression (Kazanskaya et al., 2000). Upon overexpression of *dkk1*, the PME expands and the forebrain in increased (as analysed with *Otx2*, *Bf1* and *Xanf*) but mesencephalon and hindbrain are decreased. When DKK1 function is inhibited by the addition of anti-DKK1 antibodies, PME markers *hex*, *gsc* and *blimp1* are reduced and forebrain marker *Otx2* shows a reduced expression with an expansion of the *En2* domain. Whilst the PME is expanded by overexpressed *dkk1*, it cannot be ectopically induced without BMP inhibition, indicating that both are required for formation of this tissue. The addition of *dkk1* can rescue the phenotype of embryos posteriorised by exogenous BMP4, Smad1, Smad5, Wnt3a and bFGF although *dkk1* could not rescue embryos posteriorised by exogenous RA. When *dkk1* is injected into animal caps pre-injected with BMP4, the induction of downstream targets of BMP is reduced (*Xvent1*, *Xvent2* and *sizzled*) at blastula to gastrula stages, indicating that BMP requires Wnt signalling at these stages. When the same experiment is performed but with bFGF-treated animal caps, injection of *dkk1* cannot affect the induction of FGF's target, *Xbra*, indicating that Wnt-dependent posteriorisation by FGF occurs indirectly, for example by secondary induction of Wnts (all from Kazanskaya et al., 2000).

In mouse, the mutant for *dkk1* lacks head structures rostral to the midbrain (Mukhopadhyay et al., 2001). When mutant ES cells are injected into wild-type embryos the phenotype is indistinguishable from the full homozygous mutant indicating that the anterior neural defects are not the result of a failure in AVE. This led the authors to conclude that the other site of *dkk1* expression, the anterior mesendoderm, is responsible for the phenotype (Mukhopadhyay et al., 2001). The heterozygous *dkk1* mutants have been described as phenotypically normal (Mukhopadhyay et al., 2001), as have mice mutant for *noggin* (McMahon et al.,

1998; Bachiller et al., 2000) however, double heterozygotes for these two mutations produce embryos that have no head structures anterior to the mid-hindbrain boundary (del Barco Barrantes et al., 2003). Since the AVE markers in these mutants are expressed normally with the exception of *dkk1* itself (which is down-regulated) the authors conclude that the effects are due to a knock down of Wnt- and BMP-antagonists in the anterior mesendoderm.

These results show that Wnt antagonist, Dkk1 is a very potent head inducing agent in *Xenopus* and that it is required for normal head development; both Wnt and BMP inhibition are required for the development of the PME. Dkk1 can prevent the posteriorising effects of FGF and BMP over-expression but unlike FGF, BMP signalling requires Wnt at early stages.

iii. Nodal antagonism

The Nodal family of genes, a branch of the TGFβ signalling family, plays an important role in mesendoderm induction and patterning in vertebrate embryos (Schier & Shen, 2000; Smith, 1995a;b; Whitman, 2001; Jones et al., 1995; Rebagliati et al., 1998a;b; Sampath et al., 1998). The Nodals are transmitted by the same components used by other members of the TGFβ family, like Activin receptors and SMADs (reviewed by Schier, 2003). In zebrafish the role of Nodal/Activin signalling in establishing the anteroposterior axis was investigated (Agathon, Thisse & Thisse, 2001; 2003; Thisse, Wright & Thisse, 2000). By injecting successively greater quantities of *nodal* and *activin* antagonist *antivin* (*atv*) mRNA (a Lefty/EBAF or endometrial bleeding associated factor-related factor, Thisse et al., 1999) into 1-4 cell stage embryos progressive mesendoderm deletions were observed. The lowest doses of *atv* resulted in the loss of the cephalic mesoderm, the PME and endoderm whilst higher doses also reduced more axial as well as paraxial and ventral mesoderm. Therefore, mesodermal regions were lost progressively, from anterior to posterior, as the dose of *atv* increased. The highest doses of *atv* also resulted in deletions of ectoderm in a graded, posterior to anterior manner. The authors suggest that, since the animal territory differentiates into ectodermal structures that display a large degree of correct A-P organization even when mesendodermal structures are deleted, these are not required for A-P patterning. However, although head-type structures are detected in the mesendodermally-compromised embryos, forebrain marker, *Six3* is not expressed normally and it seems that neural structures do not

have the correct morphology. These results (all from Thisse et al., 2000) show that Nodal/Activin is of fundamental importance in the generation of mesendoderm. It acts in a graded way to specify territories, the highest levels required for anterior mesendoderm and posterior ectoderm, and lower levels required for posterior mesoderm and anterior ectoderm. Further evidence for the graded effects of Nodal on the mesendoderm comes from a study by Gritsman et al (2000). In the blastula stage zebrafish embryo, the highest levels of Nodal are observed at the dorsal blastula margin where the precursors for the PME lie. Notochord progenitors reside more posteriorly. A complete absence of Nodal results in these precursor cells acquiring a neural fate. However a reduction in signalling mediated by Nodal-antagonist, Lefty, causes the PME progenitors to develop into notochord (Gritsman et al., 2000). These results show that Nodal is involved in the specification of neural versus mesendodermal and prechordal versus notochordal specification in a concentration-dependent manner.

In mouse, Nodal signalling also acts in a concentration-dependent manner, eliciting different responses at different concentrations (Robertson et al., 2003; Vincent et al., 2003). In the pre-streak mouse embryo, the level of *nodal* is initially highest proximally, at the margin with the extra-embryonic ectoderm and is later restricted to the prospective posterior of the embryo where it is required for primitive streak formation (Zhou et al., 1993; Conlon et al., 1994; Perea-Gomez et al., 2002; and in chick: Bertocchini and Stern, 2002). Nodal is required for the induction of the AVE (Brennan et al., 2001), which is induced by the lowest levels of Nodal (Robertson et al., 2003; Vincent et al., 2003). Following the induction of the AVE by Nodal (Brennen et al., 2001), Nodal is then repressed by the AVE, an effect involving the antagonists, Cerberus and Lefty1 (Belo et al., 1997; Belo et al., 2000; Meno et al., 1997) as in earlier stages of development (Perea-Gomez et al., 2002; Bertocchini et al., 20020). This may establish a further gradient in Nodal activity, being greatest in the region of the proximal-posterior visceral endoderm and lowest near the AVE. It has recently been shown that Nodal signalling stimulates the proliferation of the distal VE cells (on the prospective posterior side) resulting in the movement of the AVE towards the future anterior side (Yamamoto et al., 2004) although the AVE cells have also been shown to move anteriorly by a process of active migration (Srinivas et al., 2004). Nodal is therefore crucial in establishing the initial pattern of the vertebrate axis, acting at both pre- and post-gastrulation stages.

The Nodal antagonist, Cerberus has multifunctional activities and can inhibit BMP and Wnt signalling as well as Nodal signalling (Piccolo et al., 1999). When a construct designed for production of Flag-tagged Cerberus protein was transfected into 293T cells, two protein products were observed. One was the full length protein, termed Cer-L (long) and the other was a proteolyticaly cleaved form, termed Cer-S (short). The long form can bind BMP, Wnt8 and Xnr (Xenopus nodal related), apparently all in different places whereas the short form specifically binds to Xnr (Piccolo et al., 1999). Injection of a construct expressing the short form of Cerberus, *cer-S*, into *Xenopus* embryos at the one-cell stage caused defects in the head including cyclopia. Injection at the 4-cell stage produced embryos lacking axial structures and the trunk mesoderm markers *Xbra* and *Xwnt-8* were repressed. These results can be explained by the fact that injection of *cer-S* mRNA blocks the endogenous expression of *cerberus* in the anterior endoderm, thought to play a role in head development (Silva et al., 2003). A reduction in endogenous *cerberus* might remove inhibition from BMP, Wnt as well as Nodal signalling pathways. In combination with BMP antagonist, *tBR*, injections of *cer-S* into ventral marginal zone explants result in the formation of a large cyclopic eye, brain, cement gland and endoderm but no notochord or somites. This indicates the generation of "head organizing activity". However, it was not possible to test the effects of Wnt activity in this assay because XWnt-8, the main Wnt in the VMZ is inhibited at the gene-expression level in embryos injected with either *cer-S* or *tBR*. Therefore, BMP antagonist *chordin* and *cer-S* were injected into a ventral blastomere of Xenopus embryos, which resulted in the formation of an ectopic head possessing a cyclopic eye. When *Xwnt-8* was co-injected, this induction was blocked. Therefore, BMP, Wnt and Nodal must be inhibited to produce an ectopic head.

However, in previous experiments it was shown that head induction requires only injection of BMP and Wnt antagonist (Glinka et al., 1997), querying the role of Nodal inhibition. If the Wnt antagonist *Frzb-1* and the BMP antagonist *chordin* are co-injected, an ectopic head forms but this effect is blocked by the addition of *Xnr-1* DNA suggesting that Nodal inhibition is also required for head development (Piccolo et al., 1999). Furthermore, the authors showed that co-injection of *Frzb-1* and *tBR* resulted in strong ectopic expression of *cerberus* in the ventral endoderm. This suggests that Nodal is a head antagonist and its inhibitors are required for correct

head development although the effects of Nodal are probably acting indirectly in normal development through the induction of mesoderm (Thisse et al., 2000).

These results in *Xenopus* indicate the importance of Cerberus in head development. In mouse, the picture is less clear. Cerberus-related (*Cerr-1*) and cerberus-like (*Cer-l*) (which encode the same protein) null mutants show normal head development (Belo et al., 2000; Shawlot et al., 2000). However, the mouse Cerberus is not able to bind to Wnt, indicating that it might not have quite the same function as in *Xenopus* (Belo et al., 2000). Interestingly, wild-type presomitic/somitic mesoderm, which expresses *cerr-1,* can maintain neurectodermal expression of *Otx2* in explant culture but *cerr-1-/-* mesoderm cannot (Shawlot et al., 2000). This suggests that Cerberus might have a role in head development but a single mutant cannot uncover it.

It would seem likely that a combination of BMP, Wnt and Nodal inhibition is required both to maintain expression of neural and anterior markers in the epiblast and to go on to produce anterior neural structures. A 'two-inhibitor' model was proposed by Glinka et al., (1997) suggesting that both Wnt and BMP inhibition are required to achieve head formation. It is likely that Nodal inhibition is also required indirectly for this process (Piccolo et al., 1999; Thisse et al., 2000).

1.4.4 Development of the Caudal Neuraxis

The organizer can induce an entire rostrocaudal axis (Spemannn and Mangold, 1924). The organizer therefore has the ability to induce neural tissue that expresses regional markers from the most anterior to the most posterior points. However, organizer-derived mesoderm might also play a role in caudalisation. The role of the organizer and organizer-derived mesoderm in patterning the posterior neuraxis, as well as the signals involved in this process, will be discussed below.

i. The Node can induce and pattern a secondary axis

The signals emanating from the organizer/node clearly change with time in that a young node can induce a secondary axis that is correctly patterned along the rostrocaudal axis but nodes derived from progressively older donors lose the ability to induce more anterior markers while still inducing posterior markers (Dias and Schoenwolf, 1990; Storey et al., 1992). Furthermore, it has been shown that if

Hensen's node from 5-20 somite stage chick embryos is co-cultured with an explant of ventral neural tissue from the rostral cervical level of 5-6 somite stage embryos, it can induce within the latter different Hoxc genes (*Hoxc6*, *Hoxc9* or *Hoxc10*) depending on the stage of the node graft (Liu et al., 2001). A node taken from a 5 somite donor will induce *Hoxc6* only, a node from a 10 somite donor will induce both *Hoxc6* and *Hoxc9*, from a 15 somite donor mainly *Hoxc9* but also small amounts of *Hoxc6* and *Hoxc10* and a node from a 20 somite donor will induce *Hoxc9* and *Hoxc10* (Liu et al., 2001). This suggests that the signals emanating from node, even after the PME emerges, continue to change either qualitatively or quantitatively. However, there is also evidence from *Xenopus* that the signals from the node remain constant and act to neuralise the ectoderm and it is the signals from the non-organizer mesoderm that change and pattern the overlying ectoderm (Wacker et al., 2004); this will be discussed further below.

ii. Axial and Paraxial Mesoderm signal to the neurectoderm

Axial mesoderm taken from the roof of the invaginated amphibian archenteron was used by Mangold (1933) to show that specific A-P sections of mesoderm can pattern distinct A-P regions of the neural plate (Mangold, 1933). More recently it was shown that *Xenopus* organizers of different ages could not induce an A-P pattern in apposing ectoderm explants, as tested by regional neural marker expression (Wacker et al., 2004). The non-organizer mesoderm (taken from ventralised embryos) was also unable to induce an A-P pattern in ectoderm. However, when combined, non-organizer and organizer mesoderm could induce expression of *En-2* (mid/hindbrain), *Krox*-20 (hindbrain), *Hoxb-4* (posterior hindbrain), *Hoxc-6* and *Hoxa-7* (anterior spinal cord), and *Hoxd-13* (posterior spinal cord). The age of the organizer used in combination with non-organizer mesoderm and ectoderm did not have an effect on the pattern of genes induced in the ectoderm. However, when the non-organizer mesoderm was progressively aged, the most anterior neural genes were also progressively deleted. In these experiments, the age of the organizer was unimportant, it provided a steady neuralising signal, but the older the non-organizer mesoderm, the more posterior the Hox genes induced (Wacker et al., 2004) suggesting that it is non-organizer mesoderm, lying beneath the neurectoderm, that imparts regional information.

The paraxial mesoderm was also investigated in the chick to assess its role in caudalisation of the neuraxis. If single somites from somite (s) 1 to somite 9 of a stage 10 chick are grafted into an anterior position, adjacent to rhombomere 4, s1 to s3 never induce the posterior marker *Hoxb-4* in r4, s4 and s5 occasionally do and s6 to s9 always do (Itasaki et al., 1996). This suggests that paraxial tissue taken from a donor of the same stage can caudalise more anterior neural structures and that this ability increases as the tissue is derived from more posterior locations. Furthermore, it was shown that the ability of a given somite, say, s4, to do this decreases as the embryo develops so that, rather than just a relative difference in A-P position between graft and host site, the paraxial tissue from a given level had a caudalising potency which decreases as it ages (Itasaki et al., 1996). Therefore, paraxial mesoderm is able to caudalise the neuraxis and younger, more posteriorly located mesoderm has stronger caudalising abilities.

Anterior pre-somitic mesoderm (PSM) has also been suggested to be able to posteriorise the neurectoderm (Muhr et al., 1997; 1999; Liu et al., 2001). When explants of neurectoderm explants from stage 3 chick embryos were cultured with stage 3+ PSM, *Otx2* (forebrain), *En1/2* (mid/hindbrain) and *Krox20* (hindbrain) were induced but not *Hoxb8* (spinal cord). If stage 4 PSM is used, these markers are still induced but also *Hoxb8* expression appears at low levels. Co-culture with stage 7 PSM results in very few *Otx2* positive cells and no *En1/2* or *Krox20* but *Hoxb8* is expressed at high levels. Therefore PSM taken from progressively older donors is able to induce progressively more posterior markers when cultured with neural explants. This activity was no longer observed when using stage 10 donors. PSM harvested from varying A-P positions placed the strongest activity in the anterior PSM and most recently formed somites (Muhr et al., 1999; Muhr et al., 1997). The paraxial mesoderm clearly plays a role in refining the A-P markers in the neuraxis and is able to induce more posterior neural character as its embryonic age increases.

iii. Planar signalling through the Neurectoderm

When rhombomeric neural tissue is transplanted from an anterior position to a more posterior one, the identity of the graft is altered to that of its new position as evidenced by Hox gene expression (Grapin-Botton et al., 1995). However, when a rhombomere graft is taken from a posterior location and grafted more anteriorly its regional identity is not changed. This suggests either that neural tissue has the ability

to be caudalised but cannot be anteriorised or that patterning signals are lost in older tissue. Large grafts of hindbrain translocated to slightly more posterior positions result in *Hoxb4* being induced in the caudal part of the graft only, whereas if the graft is placed even more posteriorly, the majority of it starts to express *Hoxb4*. This suggests that the strength of caudalising signals increases at more posterior points of the axis (also noted by Itasaki et al., 1996) but also that the signal might be spreading along the neurectoderm from posterior to anterior. A stronger signal, like that provided to more caudally placed grafts, is required to spread all the way along to the anterior part of the graft. In these experiments, the question was raised as to whether the patterning signals emanate from the mesoderm beneath or whether they spread through the plane of the ectoderm. By removing the notochord, it was found not to have an influence the A-P patterning of the neural tube at hindbrain levels and it was considered that the paraxial mesoderm did not have this ability either (however this was not formally tested). These results led to the conclusion that the signals patterning the hindbrain along the A-P axis are planar and that they spread in a caudal to rostral direction. An observation that supported this was that the graft had to be placed in complete alignment with the host at both their anterior and posterior boundaries (Grapin-Botton et al., 1995). Therefore it is possible that patterning signals travel along the neurectoderm as well as vertically from the mesoderm and this will be discussed further below.

1.4.5 Molecules involved in Caudalisation

There is considerable experimental evidence for a role of FGFs in caudalisation in various vertebrates. FGFs are expressed in the node, primitive streak and presomitic mesoderm (Mahmood et al., 1995; Storey et al., 1998; Shamim and Mason, 1999; Bertrand et al., 2000; Ohuchi et al., 2000; Walshe and Mason, 2000) and are therefore present in the tissues most likely to be involved in caudalisation. When Xenopus animal caps are incubated with bFGF, in the presence of noggin, the tissue extends and expresses both the anterior marker *Otx2* and the posterior marker *Hoxb9*. When the concentration of FGF is increased, the region expressing *Hoxb9* also increases at the expense of *Otx2* indicating caudalisation (Lamb & Harland, 1995). A similar concentration-dependent effect of FGF was seen when it was applied to dissociated animal caps (Kengaku & Okamoto, 1995). At lower concentrations of

FGF, anterior neural markers, *XeNK-2* and *En2* were detected in the animal caps but these markers were repressed at higher doses, while more posterior markers were induced. Interestingly, the more anterior markers were detected after longer incubation times and the posterior markers after shorter times. Also in these experiments, ectoderm taken from older embryos, at the mid-gastrula stage, was no longer competent to express more anterior markers in response to FGF. At this stage in the embryo, the organizer mesoderm has not yet come to underlie the anterior dorsal ectoderm suggesting that an FGF signal emanating from this mesoderm might spread throught the plane of the ectoderm creating a gradient (Kengaku & Okamoto, 1995).

Additionally, chick neurectoderm explants cultured with increasing concentrations of FGF express progressively more posterior Hox genes (Liu et al., 2001). This, along with the Xenopus results (Kengaku and Okamoto, 1995; Lamb and Harland, 1995), show that FGF can posteriorise neural tissue in a concentration-dependent way.

The pathway of this caudalisation was investigated in *Xenopus* embryos, in which over-expression of eFGF leads to a posteriorised phenotype (Isaacs et al., 1994). In these embryos, the rostral and lateral extension of the *Hoxa7*, *Hoxb9* and *Hoxc6* in the neurectoderm is expanded and there is elevated expression in regions where these markers are normally expressed. The posterior markers *Xcad3* and *Xbra* are also up-regulated (Pownall et al., 1996). Conversely, if a dominant negative form of the FGF receptor is injected, *Hoxa7* and *Xcad3* expression domains are severely reduced although the anterior Hox gene, *Hoxb1* as well as the forebrain marker, *Otx2* are unaffected. To investigate the molecular pathway involved in this activity of FGF, the authors performed a series of mRNA injection experiments at gastrula and early neurula stages and analysed the results by RNAase protection. The results suggest that eFGF increases expression of *Xcad3* which, in turn, up-regulates *Hoxa7*. *Xbra* was shown not to be directly involved in this pathway. *Xcad3* can up-regulate expression of *Hoxa7* in normal embryos but it can also rescue Hoxa7 expression in embryos when FGF signalling is blocked, indicating that it acts downstream of FGF (Pownall et al., 1996).

eFGF/FGF4, as used in the previous experiment (Pownall et al., 1996), predominantly acts through FGFR1, whereas FGF8 acts via FGFR4 (Hardcastle et al., 2000). The neural inducing abilities of *eFGF* and *FGF8* were tested by injecting each into the animal pole of a *Xenopus* embryo. *FGF8* induced the neural marker, *N-*

tubulin in 91% of embryos without ectopic induction of the notochord and somite marker, *collagen type II*. Injection of *FGF8* could also repress *Xbra* expression in gastrula embryos increasingly with greater concentrations. Conversely, *eFGF* injected into the animal pole elicited *N-tubulin* expression in 51% of embryos and was able to upregulate *Xbra* strongly in gastrula stage embryos (Hardcastle et al., 2000). This suggests that FGF4 can posteriorise the neuraxis only indirectly through mesoderm induction whilst FGF8 has a direct effect on the neurectoderm. However, this is context dependent. *FGFR1* expression is detected caudally whereas *FGFR2/4* are not (Friesel and Brown, 1992; Riou et al., 1996) implicating FGFR1 in posteriorisation. FGFR1 can activate the Ras-MAPK pathway and induce mesoderm in naïve ectoderm in *Xenopus* but when it is overexpressed in neuralised ectoderm explants, it results in the caudalisation of neurectoderm, inducing *Krox20* and *Hoxb9*; hence, Ras-MAPK-mediated FGF signalling is important for neural posteriorisation (Umbhauer et al., 2000; Ribisi et al., 2000). *FGFR4* is strongly expressed in the anterior neural plate (Riou et al., 1996) and its overexpression in naïve neuralised *Xenopus* ectoderm explants induces the midbrain marker, *En2,* but not more caudal neural markers and the authors suggest that it might not activate Ras-MAPK (Umbhauer et al., 2000). This indicates context dependent roles for FGF signalling via different receptors.

In the chick, FGF8 beads can induce the caudal neural markers *Cash4* and *Sax1* in the area opaca (Storey et al., 1998). Anterior neural markers are never observed (Storey et al., 1998). Since the PSM has been implicated in caudalisation of the neuraxis (Muhr et al., 1997; 1999; Liu et al., 2001) and FGFs are expressed there, as well as in the primitive streak (Mahmood et al., 1995; Storey et al., 1998; Shamim and Mason, 1999; Bertrand et al., 2000; Ohuchi et al., 2000; Walshe and Mason, 2000) the role of FGF signalling in the PSM was also analysed (Diez del Corral et al., 2002). PSM was removed from stage 7/8 embryos and cultured for 4 hours, during which time 4 somites form on the contralateral side. In the absence of somites, the neural differentiation marker, *NeuroM* fails to be expressed in the overlying neurectoderm whilst *Sox2* is still expressed. Interestingly, after 6 hours of culture, if the PSM was still absent, premature expression of *NeuroM* was observed in the caudal neural tube. This suggests that the PSM represses neural differentiation whereas the somitic mesoderm activates it. The role of the PSM in maintaining a progenitor cell state in the caudal neural plate was analysed by implanting FGF4

beads beneath it. Caudal neural plate markers were maintained by FGF, even in neurectoderm that had already formed a neural tube, evidenced by ectopic expression of *cash4* and *Sax1* and a reduction of the neural differentiation marker, *NeuroM*. Therefore, FGF could act in the PSM to maintain an undifferentiated state in the overlying neural plate and in fact, somitic mesoderm can down-regulate *FGF8* mRNA in the PSM (Diez del Corral et al., 2002). A model has been proposed to explain this role of FGF in A-P patterning in which FGFs in the pre-somitic mesoderm continuously induce expression of the posterior/immature neural markers *Cash4* and *Sax1* in the progenitor pool of neural cells near the node (Diez del Corral et al., 2002; 2003; 2004). The FGF signal delays cells leaving this region to form part of the neural tube, thus maintaining them in the vicinity of the caudalising signals for longer (Diez del Corral et al., 2002; Mathis et al., 2001).

Wnt signalling has also been implicated in caudalisation (McGrew et al., 1997; Domingos et al., 2001; Kiecker and Niehrs, 2001; Kudoh et al., 2002; Houart et al., 2002; Nordstrom et al., 2002; Shimizu et al., 2005). Wnt proteins have been categorised into two functional groups: Wnt1 (including Wnt3a, Wnt8 and Wnt8b) and Wnt5a (including Wnt4 and 11) [reviewed in (Yamaguchi, 2001)]. The Wnt1 group can cause axis duplications when mis-expressed in Xenopus, and is thought to signal through the canonical pathway; the latter group cannot induce an axis, is thought to be involved in convergent extension and acts through the planar polarity pathway. *Wnt3a*, *Wnt5a* and *Wnt8c* are expressed in the primitive streak and emerging mesoderm as well as in the overlying posterior ectoderm in the chick (Yamaguchi, 2001).

In mouse, many mutants for the various Wnts have been created. Lowering the Wnt dosage in an allelic series for *Wnt3a* creates mutant embryos with progressively more severe trunk and tail defects and in the absence of *Wnt3a*, ectopic neural tubes develop posteriorly instead of paraxial mesoderm (Greco et al., 1996; Yoshikawa et al., 1997). In *Xenopus*, Wnts have been shown to caudalise neural tissue progressively. When animal caps are disaggregated and treated with soluble XWnt8 in increasing concentrations it leads to progressively more posterior neural gene expression (Kiecker & Niehrs, 2001). The effect was abolished by addition of the ligand binding domain of *Frz8*. Not only are posterior neural genes induced in response to activating the canonical Wnt pathway, but posterior mesodermal markers

also activated at blastula stages (Domingos et al., 2001). However it was shown in the same study that at gastrula stages, only posterior neural markers are induced, indicating both direct and indirect roles of Wnt signalling in caudalisation of the neuraxis. In the chick, and it has recently been shown that Wnts can progressively caudalise the forebrain territory (Nordstrom et al., 2002). In explant and in whole embryo culture, the application of WNT3A to the prospective forebrain acts in a concentration-dependent manner to transfrom anterior neural tissues into caudal forebrain, midbrain and finally hindbrain. In this series of experiments, FGF8 was suggested to act as a permissive factor for Wnt3a caudalisation (Nordstrom et al., 2002). Additionally, when an inducible form of ß-catenin is introduced into *Xenopus* embryos and activated it can induce posterior neural markers: an indirect non-cell autonomous effect shown to require for FGF signalling (Domingos et al., 2001). Therefore, Wnts seem to act in a concentration-dependent manner to influence A-P patterning of the neuraxis and might require FGF as a permissive factor.

It is possible that two of the signalling pathways involved in caudalisation, Wnt and FGF, interact with each other. In *Xenopus*, the early posterior expression of *XWnt3a* overlaps with *FGF3* and *eFGF* (McGrew et al., 1997). When animal caps are injected with *noggin* alone, *Xanf*-1 and *Otx2* are induced and co-injection of XFD (a dominant negative FGF receptor) produces the same result indicating that anterior neural gene expression might not be dependent on FGF signalling. If *XWnt3a* is added, the more posterior genes *En2* and *Krox20* are induced but anterior markers are not suppressed. When *FGF* is injected with *noggin*, a full range of rostro-caudal markers is induced but if a dominant negative form of *XWnt8* is included, there is an increase in anterior and decrease in posterior markers (McGrew et al., 1997). This suggests that FGF is required for Wnt to suppress anterior markers, that Wnt does not require FGF to induce posterior genes, and that Wnt is required for FGF to induce both anterior and posterior neural genes. An involvement of FGF in the ability of Wnt to suppress anterior neural markers was also noted in the chick when FGF8 was found to act permissively for WNT3A to caudalise the head territory (Nordstrom et al., 2002). However, in a study in zebrafish in which XFD, Dkk1, FGF3 and LiCl were employed in various combinations, it was found that Wnt can suppress anterior markers in the absence of FGF, that Wnt requires FGF to induce posterior markers and that FGF can suppress anterior markers in the absence of Wnt

but FGF requires Wnt to induce posterior markers (Kudoh et al., 2002). These results are rather contradictory. The differences could be due to the different factors used to over-express or block the FGF and Wnt pathways, or might reflect the difference in assays or model organisms: using explants in the *Xenopus* experiments of McGrew et al. (1997) compared to a whole embryo approach in zebrafish in the latter as well as an effect of eliciting different responses at different times.

The Wnt and FGF signalling pathways have been shown to converge on the *caudal* genes (reviewed in Lohnes, 2003). *Caudal* genes have been implicated in anterior-posterior patterning in *Drosophila*. They have been categorised as belonging to the 'parahox' group and it is thought that they share a common ancestor with the *Hox* genes. The vertebrate *cdx* genes have a role in anterior-posterior patterning but, unlike the *Dosophila* genes, in vertebrates they lie upstream of the *Hox* genes (Lohnes, 2003). Mouse mutants null for *Cdx-1* or *Cdx-2* display anterior homeotic transformations. *Hoxa7*, *Hoxc5*, *Hoxc6*, *Hoxc8* and *Hoxd3* are shifted posteriorly in the *Cdx-1* mutant (Chawengsaksophak et al., 1997; Subramanian et al., 1995). Mice expressing a transgene for *Cdx-4* show an anterior expansion of *Hoxb8* (Charite et al., 1998). In the chick, *cCdx-B* misexpression in the anterior mesendoderm leads to the activation of *Hoxa7*, *Hoxc6* and *Hoxc8* (Ehrman and Yutzey, 2001) and in *Xenopus* when *Xcad-3* is overexpressed, there are anterior truncations as well as upregulation of *Hoxa7*, *Hoxc6*, *Hoxb7* and *Hoxb9* (Isaacs et al., 1998). Therefore, members of the *cdx* family regulate Hox gene expression and these gain and loss of function experiments reveal that they act to caudalise the axis via Hox gene regulation.

FGFs, Wnts are thought to regulate *cdx* expression. Binding sites of the down-stream effectors of Wnt signalling, LEF/TCF are found in the promoter of mouse *Cdx-1* and consistent with this, *TCF4* mutant mice display a reduced expression of *cdx1* in the caudal region of the embryo (Ikeya & Takada, 2001; Lickert et al., 2000). A similar effect is seen in zebrafish when *Wnt3a* or *Wnt8* levels are reduced by morpholino injection: there is a reduction in *cdx1a* and *cdx4* expression and a loss of tail structures (Shimizu et al., 2005). The same phenotype also seen in the *cdx1a* and *cdx4* morphant embryos, suggesting that *cdx* genes mediate Wnt signalling (Shimizu et al., 2005).

FGFs have been shown to regulate *cdx* in *Xenopus* and chick. In *Xenopus*, bFGF can induce *Xcad3* whilst XFD (a dominant negative FGF receptor) causes a loss or posteriorisation of *Xcad3* expression (Isaacs et al., 1998; Pownall et al., 1996). In the chick, exogenous FGF leads to an anterior expansion of *cdxa* and *cdxb* in the neual tube (Bel-Vialar et al., 2002). Furthermore, by electroporating a dominant negative form of the Xenopus *cdx* gene into the chick neural tube, the anterior expansion of *Hox* genes by FGF is reduced indicating that, to a certain extent, FGF acts through *cdx* to regulate *Hox* expression (Bel-Vialar et al., 2002).

Wnts and FGFs are not the only signalling molecules to be involved in *cdx* regulation. A RARE (RA response element) has been found on the promoter of mouse *cdx1* and if this element removed, the expression of *cdx1* is reduced at all stages of development (Houle et al., 2003). Additionally, there is cell culture evidence that RA and Wnt might act synergistically to induce *cdx1* expression (Prinos et al., 2001). Therefore, *caudal* genes might act as a point of convergence for the three signalling pathways implicated in caudalisation.

RA can regulate *cdx* expression (Prinos et al., 2001; Houles et al., 2003) and it is implicated in anterior-posterior patterning (Blumberg et al., 1997; Chen et al., 2001; Dupe and Lumsden, 2001; Kaiser et al., 2003; Shiotsugu et al., 2004; Molotkova et al., 2005); however it is difficult to detect precisely where RA is produced or where it acts, although a study into its distribution in the avian embryo using HPLC succeeded in detecting it at low levels in posterior regions of the embryo at stage 4/5 (Maden et al., 1998). At stage 8 the levels are moderate and concentrated posterior to the first somite; by stage 10+ it is maximal in the neural tube from the anterior boundary of the first somite down to the level of the node. A high, but slightly lower level, is detected in the somites, a moderate level of RA is found in the lateral part of the embryo between the level of the first somite and the node and lower levels are detected at the level of the node and caudally as well as anterior to the first somite. No RA is found in the CNS between the forebrain and hindbrain (Maden et al., 1998). Other evidence interpreted from the complementary domains of *Cyp26*, the RA catabolising enzyme (Sonneveld et al., 1999), and *RALDH2*, an RA synthesising enzyme reveal a synthesis region in the chick corresponding to the mesoderm emerging from the primitive streak between stage 4 and 7 and a region of degradation in the anterior neurectoderm (Swindell et al., 1999). By stage 12,

RALDH2 is expressed in the somitic and anterior pre-somitic mesoderm and *Cyp26* is detected in the neural tube, initially anteriorly in the first 5-9 somites but this is displaced posteriorly though development to end at the tailbud (Swindell et al., 1999).

RA is a caudalising factor (Blumberg et al., 1997; Chen et al., 2001; Dupe and Lumsden, 2001; Kaiser et al., 2003; Shiotsugu et al., 2004; Molotkova et al., 2005). Its effects are primarily mediated through two classes of receptor: RARs and RXRs. RARs are activated by all-trians RA and 9-*cis*-RA whilst RARs are only activated by the latter (Heyman et al., 1992; Levin et al., 1992). In *Xenopus* overexpression of RA can induce a concentration dependent anterior truncation and posterior enlargement (Durston et al., 1989; Sive et al., 1990) via its effects on mesoderm and ectoderm (Ruiz i Altaba and Jessell, 1991; Sive and Cheng, 1991, Cheng et al., 2001). Low concentrations of exogenous RA lead to anterior structures being repressed and as the concentration increases, larger anterior truncations are produced (Durston et al., 1989; Papalopulu et al., 1991; Papalopulu and Kintner 1996). Furthermore, if a dominant negative form of the RARα receptor (allowing DNA binding but not transcriptional activation) is injected, an opposite phenotype to RA overexpression is observed: embryos develop with enlarged anterior and truncated posterior regions (Blumberg, 1997). In mouse and zebrafish, a reduction in RA produced by a mutation in one of its synthesis enzymes, *RALDH2*, also results in the reduction of posterior neural ectoderm and a decrease in *Hox* gene expression (Begemann et al., 2001; Grandel et al., 2002; Niederreither et al., 1999) whilst mutations in the RA degrading enzyme, *Cyp26*, in the mouse results in an expansion of posterior domains (Abu-Abed et al., 2001; Sakai et al., 2001). These data suggest that RA plays a role in caudalising the neuraxis. Over-expression of RA results in a posteriorised embryo whereas a reduction in its signalling causes anteriorisation.

The targets of RA signalling have been investigated and it has been shown that 3' *Hox* genes are sensitive to this factor, whilst more caudally expressed 5' *Hox* genes are sensitive to FGFs (Bel-Vialar, 2002). Furthermore, it was shown that the *Hox* gene expression in non-organizer mesoderm in Xenopus can be translated to neurectodermal expression of that *Hox* gene and that RA is required for this signalling process (Molotkova et al., 2005). Hence, RA appears to act to transfer A-P positional information in the mesoderm, vertically, to the overlying neural plate. The role of RA was also investigated in the somitic mesoderm in follow-up experiments

to those by Diez del Corral et al (2002) on FGF in the presomitic mesoderm. The RA synthesising enzyme, *RALDH2*, is expressed in the somitic mesoderm and the anterior portion of the presomitic mesoderm whilst RA receptors, *RXRα*, *RARα*, *RARβ* and *RARγ* are expressed in the spinal cord and, in the case of the latter, the caudal neural plate (Diez del Corral et al., 2003). Caudal neural plate explants were cultured in the presence of RA or an RAR agonist. The explants upregulated expression of neural differentiation marker, *NeuroM,* in the presence of these factors (Diez del Corral et al., 2003). The data above suggest that RA signalling from the somitic mesoderm encourages neural differentiation of the caudal neural plate and also that it can translate Hox gene information from the mesoderm to the neurectoderm.

Both FGF- and Wnt-signalling have been shown to interact with RA in patterning the neuraxis. In the chick, experiments (discussed above) suggest that FGF, in the presomitic mesoderm, maintains an undifferentiated state in the neighbouring neurectoderm (Diez del Corral et al., 2002). *FGF8* expression is reduced, and consequently FGF8 signalling, by the somitic mesoderm (Diez del Corral et al., 2002). The somitic mesoderm, therefore, promotes neural differentiation and RA was found to mimic this effect (Diez del Corral et al., 2003). The somitic mesoderm seems to enable neural differentiation by blocking FGF signalling. It was shown that the expression of *FGF8* in caudal neural plate or presomitic mesoderm explants is blocked when cultured with RA or an RA agonist. These results suggest that RA does act to inhibit FGF signalling at the pre-translational level. In the converse experiments, explants of caudal presomitic mesoderm, which normally start to express *RALDH2* after 2 hours, were cultured with FGF8. Under these conditions, the expression of *RALDH2* was barely detected (Diez del Corral et al., 2003). This shows that there is mutual inhibition between RA and FGF in patterning the axis whereby *FGF8* is expressed in the presomitic mesoderm and maintains neural progentior cells, potentially in the vicintiy of a caudalising signal, whilst RA acts to promote neural differentiation.

In other chick explant experiments, in which pieces of neurectoderm were cultured with RA and or FGF, it was shown that RA can induce the more anterior *Hox* gene, *Hoxc5* while FGF induces the more posteriorly expressed *Hoxc8, Hoxc9* and *Hoxc10* genes in a concentration dependent manner (Liu et al., 2001). FGF and RA together

induce *Hoxc6* which is expressed in intermediate regions. In this study it was also shown that RA could suppress the more posterior *Hox* genes and FGF the more anterior ones. This indicates that relative amounts of RA and FGF signalling provide positional information for patterning the neurectoderm. In a thorough series of experiments in the zebrafish, it was shown that *Cyp26* can prevent FGF from inducing posterior marker, *Hoxb9* suggesting that FGF requires RA for this process, but that RA does not require FGF signalling for it to induce these posterior genes (Kudoh et al., 2002). This indicates that RA might be downstream of FGF signalling. Shiotsugu et al. (2004) showed that blocking FGF signalling in Xenopus reduces expression of *Xcad3* and *Hoxb9* but that this effect could be rescued by adding exogenous RA. This result supports the idea that RA is downstream of FGF in the induction of posterior neural gene expression. In summary, FGF and RA can mutually antagonise each other to determine neural progenitor verus neural differentiation state. They also act to specify *Hox* gene identity in the neurectoderm although the mechanism of this is not entirely clear.

There is also evidence for interactions between Wnt and RA signalling. The canonical Wnt signalling pathway requires β-catenin to binds to LEF/TCF (Easwaran et al., 1999). However, 9-*cis* RA results in increased cell-cell adhesion and the recruitment of cytoplasmic β-catenin to the membrane. The RA receptor RAR is able to bind to β-catenin as well as reducing the binding of LEF/TCF to β-catenin in GST pull-down experiments (Easwaran et al., 1999). This suggests a competition between Wnt and RA signalling, where RA might be a rate-limiting factor. These experiments suggest a potential negative regulation of RA on Wnt signalling but this has not been tested with respect to caudalisation of the neuraxis. In the zebrafish, it was suggested that Wnt and RA act independently whereby Wnt only indirectly affects RA signalling by specifying the tissue that will later express *RALDH2* (Kudoh et al., 2002).

Another set of genes that could have a downstream role in neural posteriorisation are the *polycomb* genes. Initially characterised in *Drosophila*, *polycomb* genes are required for the stable repression of *Hox* genes during development by forming large multimeric protein complexes that alter the local chromatin structure (Paro, 1995; Pirrotta, 1997). In vertebrates, the *Ring1B* mouse null has a derepression of *Hoxb4*,

Hoxb6 and *Hoxb8* in cells anterior to their normal boundary in the neural tube and overexpression of *Ring1b* in chick leads to a posterior shift in the boundary of *Hoxb9* (Suzuki et al., 2002). The mouse mutant for the polycomb gene, *M33* displays a derepression in *Hoxa3* but not *Hoxc6* or *Hoxc8* in the neural tube (Core et al., 1997). *Geminin* has recently been suggested to have polycomb-like activity and can regulate the boundary of *Hoxb9* expression in mouse (Luo et al., 2004). It transiently associates with the polycomb complex that represses *Hox* expression as well as associating with the chromatin of *Hox* regulatory elements and with Hox proteins themselves (Luo et al., 2004). In Xenopus, the polycomb gene *XPcl2* overexpression results in a posterior shift in *Krox20* and *Hoxb9* (Kitaguchi et al., 2001) and inhibition of another polycomb gene, *YY1* causes a reduction in *En2* but has no effect on *Hoxb9* (Kwon & Chung, 2003). These data reveal that in vertebrates, polycomb genes seem to act to repress *Hox* gene expression, although, as shown in the results above, different polycomb genes appear to have effects specific to distinct A-P regions of the neural tube.

Polycomb genes are thought to act after the initial phase of *Hox* gene induction in the neurectoderm (Paro, 1995; Pirrotta, 1997). The zebrafish polycomb gene, *ph2α* is regulated by FGF. When FGF signalling is inhibited using SU5402 the expression of *ph2α* is markedly reduced. However, exogenous FGF does not result in an increase of *ph2α* transcripts (Komoike et al., 2005). This indicates that FGF signalling might be required for polycomb gene expression but the relationship is not a simple one. Whereas FGF is required for the expression of polycomb genes, *p2hα* (Komoike et al., 2005), it would appear that polycomb genes can affect RA targets by affecting the window of responsiveness of Hox genes to RA (Bel-Vialar et al., 2000). In the mouse mutant for polycomb gene *M33*, this window is opened earlier for *Hoxd4* and also *Hoxd11* is induced precociously suggesting that *M33* normally antagonises RA to delay its activation of Hox genes. It has been proposed that *M33* acts by controlling the accessibility of the RAREs in the *Hox* gene regulatory regions. Interestingly, later in development the expression of these genes reverts to their correct boundaries (Bel-Vialar et al., 2000).

Taken together, these results reveal that FGF, Wnt and RA are all involved in caudalisation of the neuraxis. Downstream targets of these pathways include the *caudal* and *polycomb* genes, which regulate the expression of *Hox* genes to specify A-P identity.

1.6 Timing: Neural Induction versus A-P Patterning in the Chick

In the chick, the onset of expression of *Sox2* identifies the first definitive neural tissue to be specified (Rex et al., 1997; Sheng et al., 2003). This occurs at stage 4+, a time when the PME and head process are emerging from the tip of the streak. Although the effects of neural induction are observed at 4+, it can be assumed that the process leading up to this induction started several hours previously. To support this idea, the tip of the primitive streak from a stage 2 embryo is capable of inducing neural tissue (Storey et al,1992), suggesting that the signals required to start the cascade towards neural induction are evident at least at this time. When a node from an embryo up to stage 4 is grafted into the area opaca, it takes 9 hours for *Sox2* to be induced, but other markers are induced earlier: *ERNI* (Streit et al., 2000) is induced after an hour or so, *Sox3* (Streit et al., 1998) and *Otx2* after 2-3 hours and *Churchill* (Sheng et al., 2003), after 4-5 hours. These markers could be considered 'pre-neural' because they are all induced in sequence leading up to full neural induction but cells expressing the earlier markers including *ERNI* and *Sox3* can give rise to cells in all three layers of the embryo (Sheng et al., 2003). *Otx2*, a rostral neural marker at later stages, has an early phase of expression that covers most of the epiblast and does not mark all prospective neural cells (Bally-Cuif et al., 1995) and *ERNI*, whilst involved in the pathway leading towards neural induction, appears to act as an antagonist of neural differentiation and its downregulation initiates expression of *Sox2* (Papanayotou et al., in preparation). The expression of genes induced by a grafted node is lost if the node is removed before 13 hours' contact with the area opaca and the epiblast cells become non-neural again (Gallera, 1971). These findings suggest that the process of neural induction is a long one, spanning perhaps 9 hours until the onset of *Sox2* expression and even longer before the cells become committed. It must be pointed out that node grafts placed in the area pellucida result in a faster induction of *Sox2* but this might reflect that signals previously received by the epiblast have

already specified it to a 'pre-neural' state, as they already express *Sox3* and *ERNI*. The hypoblast can transiently induce the pre-neural markers, *Sox3*, *Otx2* and *ERNI* when grafted in the area opaca (Foley et al., 2000; Streit et al., 2000) and therefore this tissue could play a role in preparing the epiblast for neural induction.

Another study looking at the timing of neural induction has also suggested that the epiblast possesses the ability to differentiate into neurectoderm prior to streak formation (Wilson et al., 2000). Pieces of epiblast taken from prestreak embryos were cultured in isolation for 40 hours and analysed for SOX2. SOX2 was detected in "medial" explants taken from the centre of embryos at stage IX and XII but not from "lateral" explants or from embryos younger than stage IX. These findings have been taken to suggest that the centre of the prestreak epiblast is specified to a neural fate even before formation of the primitive streak and node. There are a few potential problems with these interpretations. The first is that the pieces of epiblast might have been cultured along with the underlying hypoblast, which expresses *FGF8* (Streit et al., 2000), which can induce pre-neural genes such as *Sox3* (Streit et al., 1998). Indeed, Wilson et al. (2000) did detect both *FGF3* and *FGFR2* in both medial and lateral explants of stage XII embryos both before and after culture. Secondly, the expression of *Sox2* mRNA was not tested but, instead, SOX2 protein was assayed with a polyclonal antibody raised against mouse SOX2, which might cross-react with chicken SOX3, which is known to be expressed in the epiblast at prestreak stages (Kamachi et al., 1995; Avilion et al., 2003); the specificity of the anti-SOX antibodies was not tested by Wilson et al. (2000).

Darnell et al. (1999) addressed the timing of neural specification using an assay in which chick embryos were bisected rostral to the tip of the primitive streak and the anterior portion cultured. At stage 2 to early stage 3, the rostral portions of the embryo started to express neural markers, including *Sox2*. At mid-stage 3, the rostral isolates no longer expressed *Sox2* but this ability returned when the isolates were taken from embryos of late stage 3 and stage 4. These results were interpreted by the authors as suggesting that before mid-stage 3 there remained cells rostral to the tip of the streak that would be incorporated into the streak that would result in neural induction when cultured in the rostral isolates. By mid-stage 3, all these cells had been assimilated into the streak and therefore the rostral explants were not induced to express *Sox2*. At late stage 3 and stage 4, the epiblast anterior to the node has

received neural inducing signals from the node and so, when cultured alone, *Sox2* expression does result.

In conclusion, neural induction seems to be a long process starting before primitive streak formation and lasting several hours and involves a cascade of genes being expressed before a definitive neural state is induced, and even longer before these cells become committed. The end of neural induction could be defined by the stage at which neural-specified territories become committed to this fate. The inducing ability of the node decreases from stage 4+ and is completely lost by stages 6-7 (Storey et al., 1992; Streit et al., 1997). However, when stage 5 PME and node are grafted together into the area opaca, the lost ability of the node to induce forebrain markers is regained (Foley et al., 1997) suggesting that, in the normal embryo, the node and node-derived axial mesendoderm might still communicate to induce neural tissue. Also, head process taken from stage 6/7 embryos can induce neural structures in the area opaca indicating that neural induction might continue at these stages at least in anterior regions of the embryo (Rowan et al., 1999). However, the competence of area opaca ectoderm to respond to neural inducing signals from a young node is lost suddenly at stage 4+ (Gallera, 1964; Gallera, 1971; Dias and Schoenwolf, 1990; Storey, 1992; Streit et al., 1997) and it is possible that neural induction is completed by this time except in cells that have already received some preparatory signals, as marked by expression of *Sox3*, *ERNI* and/or *Otx2*.

When does A-P patterning of the neural tissue begin? Two steps can be defined by expression of early markers, which also correlate with the proposed timing of initiation of and end of neural induction. Firstly, there is an induction of a pre-neural, pre-forebrain state in the epiblast, before streak formation, by the hypoblast (Foley et al., 2000, Streit et al., 2000). This is thought to be 'anterior' (or 'pre-forebrain') in character because, although the markers are expressed throughout the epiblast and cannot simply be marking a forebrain territory, *Otx2* later becomes restricted to the anterior (prospective forebrain and midbrain) epiblast as the hypoblast moves rostrally (Bally-Cuif et al., 1995). These observations fit the 'activation-transformation' model: the earliest responses to neural inducing signals are marked by expression of anterior neural markers (*Sox3* and *Otx2*), corresponding to the "activation" step (Nieuwkoop and Nigtevecht, 1954). The second 'phase' is the induction of a definitive anterior neural state, perhaps by the precursors of the PME

and head process residing within the tip of the streak and node at stages 2-4 and later, by the emerged PME at stages 4+/5 (Dias and Schoenwolf, 1990; Storey et al., 1992; Foley et al., 1997; Pera and Kessel, 1997). These later stages of anterior neural specification seem to occur around the time that neural induction is ending, at around stage 4+.

The induced neurectoderm is subsequently caudalised. When cells that reside in the epiblast lateral to the node at stage 3 are labelled with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) they are found to populate the entire A-P neuraxis caudal to the forebrain (Muhr et al., 1999). However, if these cells are cultured for 24 hours, they express rostral markers OTX2 and neural marker SOX2 but not more caudal neural markers (EN2, KROX20, *Hoxb8*). By stage 3+, epiblast explants isolated from the region lateral to the node have OTX2, EN2 and a few KROX20 positive cells following culture and explants taken from embryos between stage 3+ and 4, express few OTX2/EN2-positive and many KROX20 positive cells. Cells isolated from a more rostral position at this stage did become OTX2/EN2-expressing following culture. Finally, at stage 4, explants were taken from four different A-P positions, the more rostral two taken from points anterior to the node, the third from a position lateral to the node, and the fourth from a region just caudal to the third. The most rostral explant produced cells expressing OTX2 only, indicating forebrain identity, whilst the next most rostral explant generated cells expressing both OTX2 and EN2 but not more caudal markers, indicative of fore/midbrain character. The explant taken from a position lateral to the node expressed mainly KROX20 but a few cells expressed *Hoxb8* and no cells being positive for anterior markers. The most caudal explant expressed both KROX20 and *Hoxb8* (Muhr et al., 1999). These data suggest two things. Firstly, the fact that the epiblast lateral to the node at stage 3 has a forebrain character even though it will later contribute to all levels of the neuraxis caudal to the forebrain indicates that the neurectoderm initially has an anterior character and this is subsequently caudalised. Secondly, the A-P identity of the epiblast is specified by stage 4 suggesting that crude rostrocaudal patterning of the neuraxis has finished by this stage (more or less coincident with the end of neural induction). The caudalisation of the epiblast between stage 3 and 4 is proposed by the authors to be a result of signals emanating from the node and nascent paraxial mesoderm. However, despite the specification of

these cells at early stages, it does not indicate a commitment. Neural progenitors residing near the node give rise to cells in the hindbrain and along the length of the spinal cord (Mathis and Nicolas, 2000) and therefore must refine their regional identity accordingly. Both mesoderm underlying the neural progenitors and subsequently adjacent to the neural tube have been shown to influence A-P neural identity (Itasaki et al., 1996; Muhr et al., 1997; 1999; Liu et al., 1999; Diez del Corral et al., 2002; 2003; Wacker et al., 2004; Moloktova et al., 2005; Delfino-Machin et al., 2004). Therefore, although an initial specification of an A-P pattern could be established by stage 4, caudalisation and refinement is likely to continue.

The node as well as neighbouring regions of the epiblast maintain a pool of undifferentiated 'stem' cells which continuously leave to lay down the axis; this includes a "stem zone" from which the caudal neural plate will arise (Selleck and Stern, 1991; Mathis et al., 2000; Diez del Corral et al., 2002; 2003). Even once the neuraxis has been specified and subdivided into cells marking different A-P levels, its plasticity to respond to caudalising signals remains for much longer; however it would seem that it is refractory to anteriorisation.

In normal development, the initial anterior boundaries of *Hox* gene expression in the neuraxis and underlying mesoderm are not the final ones; they will be refined as the axis extends so that the neural boundary will eventually encompass cells anterior to the level of those initially expressing the *Hox* gene and the mesoderm boundary will regress so that cells at the anterior point in which there is initial expression, lose this (Forlani et al., 2003). This plasticity of the neuraxis has been experimentally analysed. The addition of retinoic acid (RA) to the hindbrain of head-fold and older stage chick embryos can induce the expression of more caudal *Hox* genes (Marshall et al., 1992). Also, reciprocal transplants of posterior and anterior tissue reveals that a graft of more anterior tissue can be transformed when placed in a more posterior position whereas in the reverse experiment, grafts of posterior neural tissue retain their identity when placed in more anterior positions, a regional plasticity that remains in the hindbrain until around stage 10 (Alvarado-Mallart, 1993; Bally-Cuif et al., 1992; Grapin-Botton et al., 1995; Itasaki et al., 1996; Martinez, Wassef & Alvarado-Mallart, 1991; Nakamura et al., 1986). Therefore, anterior regions are plastic in response to posteriorising signals but with age, this plasticity is attenuated. The ability to be converted to a more posterior character seems to persist for longer

in the caudal neural tube, suggesting that most recently formed tissue is more plastic. The polycomb-like gene, *geminin*, can shift the anterior boundary of *Hoxb9* anteriorly in the chick when electroporated between stage 9-11 and analysed at stage 18-19 (Luo et al., 2004). The caudalising effect of *geminin* must therefore be able to act after stage 11 and therefore plasticity in the neural tube must exist after this point. When the polycomb gene *Ring1B* is electroporated into the neural tube of a 6-12 somite chick embryo, an anterior shift is *Hoxb9* expression is observed. This shift is still evident when a 12-18 somite embryo is electroporated although it is not so extensive and no shift is observed when 18-24 somite embryos are electroporated (Suzuki et al., 2002). This suggests a declining ability of the anterior boundary of *Hoxb9* to respond to caudalising signals although it might indicate that *Hoxb9* is no longer responsive to that particular polycomb gene at later stages. The plasticity of the neuraxis to posteriorising signals does diminish with time, but this is not dependent on the specific embryonic stage, but rather on the age of the particular section of neural tissue being analysed, with most recently formed regions having the greatest plasticity. When compared to neural induction, in chick it would appear that neural induction is completed and anterior neural regions specified at around stage 4+. However, the process of caudalisation and the responsiveness of the neuraxis to caudalising signals persists for much longer as regions caudal to the hindbrain are laid down.

1.5 History of A-P patterning research

Many models have been proposed to explain anteroposterior patterning of the vertebrate neuraxis. These are divided into 'qualitative' and 'quantitative' models (Saxen, 1962). Qualitative models propose that there are regional differences in the mesendoderm underlying the neurectoderm and it is these differences that establish an A-P pattern in the neuraxis. Quantitative models are based on a gradient of two or more graded morphogenetic signals with each position on the embryo having a unique coordinate which is then translated into a specific A-P identity in the nervous system.

1.5.1 Qualitative Models

i. Separate Organizers

Following the initial discovery of the organizer by Spemannn and Mangold in 1924, its inducing abilities were further investigated by Spemannn and his contemporaries. When the dorsal lip of the blastopore taken from amphibian embryos ranging from early to late gastrula stage was grafted into a host, it was found that young lips could induce the full range of anteroposterior regions in the secondary axis whereas the older lips could only induce posterior structures (Spemann, 1931, 1938; Holtfreter, 1936, 1938). This led to the proposal that the mesoderm emerging from the dorsal lip at different times has the ability to induce distinct parts of the axis. The first mesoderm to involute is prechordal, suggesting that this is responsible for inducing the anterior neuraxis. Dorsal lips grafted after the prechordal mesoderm has involuted are no longer able to induce the most anterior neural structures. The population of cells that will form the trunk mesoderm remain in the dorsal lip and will subsequently induce more posterior structures (Spemann, 1931, 1938; Mangold, 1933; Holtfreter, 1936, 1938). This is also true in the zebrafish (Saúde et al., 2000). Mangold (1933) took this experiment further and looked at mesoderm that had already involuted. He removed the archenteron roof and divided it into sections along the A-P axis. These were then grafted into the blastocoele of a host embryo. Anterior regions of mesendoderm induced rostral neuraxis whilst increasingly posterior grafts induced progressively more caudal regions of the axis (Mangold, 1933) (Fig. 1.2). However, the neural structures induced by grafts of archenteron roof are not quite those expected by fate maps and sometimes more anterior regions would be induced than the fate maps would suggest (Mangold, 1933). Ter Horst (1948) studied the archenteron roof in co-cultures with two pieces of competent ectoderm removed from early gastrula stage embryos. Sections of the archenteron roof from various A-P levels have specific regional inducing abilities but again the structures induced tend to be more anterior than expected from the fate maps. Therefore, the vertical induction of regional neurectoderm via mesoderm is not so straightforward.

Based on the data from amphibians summarised above, the qualitative model for regionalisation suggests that the organizer at full inducing stages comprises distinct populations of cells that will each go on to induce particular A-P regions in the

neurectoderm and that these populations leave the dorsal lip in sequence, with most anterior inducers leaving first. Also, these populations of cells do not just induce and pattern the neurectoderm whilst in the organizer territory but rather, they continue to pattern the overlying ectoderm after involuting. There is evidence in other vertebrates for qualitative signalling. In the mouse, anterior notochord can induce the expression of engrailed (mid/hindbrain) but posterior notochord cannot (Hemmati-Brivanlou et al., 1990). In the chick, once the head mesendoderm, containing the PME, leaves the node, the node can no longer induce a secondary axis expressing forebrain neural markers (Dias and Schoenwolf 1990; Storey et al., 1992) but its ability to induce anterior neural markers can be rescued by grafting it along with a piece of PME (Foley et al., 1997). This shows that the PME is required to induce and/or pattern a distinct part of the neuraxis as in amphibians: the most anterior region and in agreement with the qualitative model, it is the first mesendoderm to emerge from the node. Other data in the chick suggest that the chordamesoderm caudal to the PME, the head process, induces specific regional character in the neurectoderm (Rowan et al., 1999). Furthermore, when grafted into the area opaca, rostral head process can induce a neural structure that expresses a specific range of fore/midbrain markers whilst caudal head process induces hindbrain/anterior spinal cord markers (Rowan et al., 1999). This suggests that axial mesoderm from different A-P positions can signal vertically to specify distinct regions of the neuraxis. However, the induced structures do not possess exactly the regional character one might expect, contrary with the results of Mangold (1933) and Ter Horst (1948), they are more posterior (Rowan et al., 1999). Paraxial mesoderm might also be involved in the A-P patterning of the neural tube (Itasaki et al., 1996; Muhr et al., 1997; 1999; Liu et al., 2001; Diez del Corral et al., 2002; 2003). When explants of presomitic mesoderm from progressively older embryos are cultured with explants of neural tissue they induce neural expression of increasingly posterior *Hox* genes (Muhr et al., 1999). This suggests that signals from the mesoderm continue to pattern the neural tube at spinal cord levels. In *Xenopus*, when explants of non-organizer mesoderm (NOM) of different stages are cultured with the organizer and a piece of neurectoderm, the *Hox* genes expressed by the NOM are induced in the neurectoderm (Wacker et al., 2004). The authors suggested that the *Hox* genes in the NOM are induced in a temporal co-linearity sequence. Their expression is intrinsically transient and requires a signal from the organizer to stabilise them.

Convergent extension continuously changes the population of cells in the vicinity of the organizer and as new NOM leaves the dorsal lip region it patterns the overlying neurectoderm. To summarise, there are data in mouse, chick and amphibians that suggest that signalling from the mesoderm can impart A-P regional identity in the neurectoderm.

The 'separate organizer' model was crystallised by work mainly done by Toivonen (1938, 1940). This model was based on the study of many different heterologous inducers and the finding that they fell into two categories: they could either induce head or trunk/tail. Therefore, there could be a series of separate organizers in the embryo, each inducing a specific A-P region of the neuraxis. Not only have separate head and trunk organizers been suggested but also a specific tail organizer in the zebrafish (Agathon et al., 2003). If the ventral margin of early blastula to mid-gastrula staged zebrafish embryo is grafted into an early blastula stage host it results in the induction of an ectopic tail that contains graft as well as host derived cells (Agathon et al., 2003). It is not clear whether the ventral margin can be regarded as a true organizer because it was not formally ruled out that host-derived cells in the ectopic tail structures might have been recruited from the host axis.

The idea of a separate head organizer has regained popularity recently with the discovery that the extra-embryonic anterior visceral endoderm in mouse is required for head formation (Thomas & Beddington, 1996) and the finding that grafts of the mouse node induce a secondary axis lacking a forebrain (Beddington, 1994). This prompted the suggestion that mammals have developed a unique mechanism for head induction whereby a head organizer exists and is distinct and separate from Spemannn's organizer (Knoetgen et al., 1999a; 1999b; 2000). The role of the AVE in A-P patterning has been discussed above with the conclusion that, although necessary for head development, it is unable to induce anterior neural markers when grafted alone to an ectopic site that has not previously received neuralising signals (Tam and Steiner, 1999).

1.5.2 Quantitative Models

i. Two-Signal model

Dalcq and Pasteels (1937) investigated the formation of the blastopore by placing amphibian embryos under glass. In this manner, they could orient them to reverse the relative position of the animal and vegetal poles. Using this technique they could cause the blastopore lip to form in any location, and occasionally, generate a second blastopore. This demonstrated that any point on the egg surface where the vegetal yolky mass cells contacts the outer cortex could result in formation of a dorsal lip and therefore gastrulation movements. However, the dorsal side was most likely to form a lip. To explain their results, they proposed the 'two-signal' model whereby there are two opposing morphogenetic gradients. The first is a vitelline gradient (V), the highest level of which is in the vegetal side and the second, (C), which is greatest in the grey crescent of the dorsal side. From this, each point of the embryo could have a co-ordinate (CxV) defined by a specific amount of each gradient or 'morphogenetic potential'. This resulted in some regions having the same morphogenetic potential. To avoid this, it was proposed that cells measure the ratio of C/V such that regions with high C/V values would become the most dorsal/anterior and as the ratio decreased, cells would become increasingly posterior/ventral. Thus, two opposing gradients could, in theory, specify an entire embryo. However it is not easy to understand how A/P patterning is separated from D/V patterning in this model.

ii. Double Potency model

This model was proposed to account for the problem that A/P and D/V patterning cannot overlap completely. Its principles are similar to those of the 'two-signal' model, of which it is a modification. It is based on a series of experiments (Yamada, 1940; 1950) in which competent ectoderm was combined with a heterologous inducer (a protein extracted from ox muscle used after various treatments with or without iron powder). The relative inductive frequency of neural and mesodermal derivatives was compared with the total inductive ability of the inducer to produce an optimal value of induction for each structure. The data that led to the 'two-signal' model (Dalcq and Pasteels, 1937) was combined with this idea to produce the 'double potency' model. Two gradients (termed Pcc: the cephalocaudal potential and

Pdv: the dorsoventral potential) existed, the opposition of which could generate positional information (Yamada, 1940). This model was later modified to account for new information indicating that morphogenetic movements were involved in the ability of the archenteron roof to induce regionalised, neural tissue (Okada & Takaya, 1942; Okada, 1942). The Pcc gradient was abandoned in favour of the Mcc (mediator of cephalocaudal potential), which was not a signal but a potential for movement of cells. The Mcc opposed the Mdv (mediator of dorsoventral potential), which was a stationary signal towards or away from which cells would be directed by Mcc, thus regulating the strength of the signal they received and the amount of time they spent in its vicinity. This specific information each cell received could impart on it a position in the rostocaudal axis (Yamada, 1950).

iii. Activation-Transformaiton model and Planar Signalling

The most famous of the quantitative models is that of Nieuwkoop and Nigetvecht (1954). They performed experiments in which they grafted folds of ectoderm perpendicularly into the neural plate of late gastrula and early neurula hosts. The regions of the graft differentiated according to their closeness to the host axis. The points furthest away remained undifferentiated, intermediate regions differentiated into ectoderm and mesoderm and the closest part formed patterned neural tissue. Within the ectopic neural tissue, there was pattern that was also dependent on the proximity to the host axis. Most proximally, it formed the most caudal neural stuctures, of either the same A-P character as that point on the host or slightly anterior, and most distally it formed rostral structures with the intermediate regions patterned appropriately. This led Nieuwkoop and Nigtevecht (1954) to two conclusions. Firstly, they proposed that ectoderm must first be induced to form anterior neural tissue, or 'activated', which is subsequently posteriorised, or 'transformed' and secondly, that the signal responsible for the transformation spreads along the ectoderm in a planar fashion. These two principles were translated into a two-gradient model. The first gradient was one of "Activation". This was based on how the position along the host embryo of the graft site related to the quantity of neural tissue formed from the graft. It was found to be highest just posterior to the prechodral/chordal boundary and the gradient declined both anterior and posterior to this. It was proposed that this gradient is dependent on the level of neural-activating activity in the dorsal mesoderm underlying the neurectoderm in these regions. The

second gradient is one of "Transformation", which could be either a gradient of increasing signal strength, or of time spent next to a steady signal. This gradient increased almost linearly from a level of zero at the anterior to the highest levels in the most posterior of the embryo which was based on the observation that grafts placed in the most posterior regions often lack anterior-most structures at their distal ends (Nieuwkoop, 1952; Nieuwkoop, 1954). It is interesting that these grafts did not develop the most rostral structures. Similar results have been observed in other species: grafts of older *Xenopus*, avian, mouse or zebrafish organizers can induce posterior neural structures lacking a head (Mangold, 1933a; Gallera, 1970; Gallera, 1971; Gallera & Nicolet, 1969; Dias and Schoenwolf 1990; Storey, 1992; Thomas & Beddington, 1996; Saúde et al., 2000). This suggests that ectoderm can be induced to form a regionalised neural structure without firstly 'activating' it. Nieuwkoops's (1954) data could be re-interpreted to accommodate this if it is postulated that the requirement for an 'activation' signal went to zero for neurectoderm at a distance from the PME/head process and that another neuralising signal, produced by the organizer, remains constant. Such a signal has been proposed by Wacker et al. (2004) to emanate from the organizer in *Xenopus* and in chick, it has been suggested that signals in the PSM, including FGF, are required to maintain a 'stem zone' of neural progenitors from which the spinal cord will arise (Delfino-Machin et al., 2005).

Although others had suggested a role for planar signalling in neural induction (Spemannn, 1924; Goerttler, 1926), the 'activation-transformation' model was new in suggesting that planar signals, spreading through the presumptive neurectoderm are involved in A-P patterning. This was based on similar grafting experiments by Nieuwkoop et al. (1952) who showed that neural structures could be formed from the graft at a distance from the inducing activities of the dorsal mesoderm. These results were supported by recombination experiments of archenteron roof and ectoderm by Sala (1955). Further support for planar signalling comes from experiments in Xenopus using Keller sandwich cultures. Keller sandwiches involve the combination of presumptive neurectoderm and dorsal mesoderm, taken prior to involution, so that vertical contact between the two tissues is prevented (Keller & Danilchik, 1988). In a single Keller explant, *En-2* (mid/hindbrain), *XKrox-20* (hindbrain) and *Xhoxb9* (posterior spinal cord) have all been observed and are expressed in the correct A-P sequence (Doniach, 1992; Doniach et al., 1992). The observation of forebrain

markers *X-dll3* and *XANF-2* in Keller sandwiches (Mathers et al., 1995; Papalopulu and Kintner, 1993) suggests that all rostrocaudal markers can be induced through planar signalling. However, not all A-P structures can be induced by planar signalling in Keller explants. Eyes are never observed when vertical signalling is prevented but if anterior dorsal mesoderm is placed in vertical contact with presumptive anterior neurectoderm in Keller explants, eyes do develop (Dixon and Kintner, 1989). *Hoxb1* can also be induced in Keller sandwich experiments but when the signals are planar, the expression is diffuse and overlapping with that of *Krox-20* (Poznanski and Keller, 1997). If vertical signalling is introduced, the expression of *Hoxb1* becomes restricted, and no longer overlaps with other markers, suggesting that in the embryo, the underlying mesoderm is required to refine the A-P pattern (Poznanski and Keller, 1997).

Exogastrula experiments, in which amphibia are placed in a hypotonic solution resulting in the dorsal mesoderm moving out into the culture medium rather than involuting, are thought to allow embryos to develop in the absence of vertical signals. This technique was used by Holtfreter (1933) to show that embryos could not form neural tissue without vertical signals. However, in some studies, these embryos can have a patterned neurectoderm, for example, *Xhox3* expression is observed in the apical ectoderm of total exogastrulae, a region that develops without axial mesoderm (Ruiz i Altaba, 1990).

In zebrafish, there is further evidence for planar signalling. *Squint* and *cyclops* (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998a,b; Sampath et al., 1998) encode different Nodal homologues and when double mutants of these are produced the resulting embryos lack mesoderm but seem to possess patterned neural tissues (Feldman et al. 2000, this is also found in the mouse *cripto* mutant (Ding et al., 1998; Liguori et al., 2003). Also, neural structures develop when mesodermal tissues are depleted by antagonising Nodal/Activin signalling using Antivin (Thisse et al., 2000). This suggests that vertical signals are not required to establish the basic A-P pattern in neurectoderm. Planar signals do not necessarily emanate only from the organizer. In zebrafish, ANB cells (located at the anterior boundary of the neural plate) can induce telencephalic markers when transplanted to presumptive caudal neurectoderm (Houart et al., 1998). These cells produce *tlc,* a Wnt antagonist that acts non-cell autonomously in a concentration-dependent manner to inhibit Wnt signalling and therefore promote telencephalic fate (Houart et al., 2002). Also,

rhombomere transposition experiments in chick revealed that planar signalling might have a role in extablising Hox gene identitiy in the hindbrain (discussed above, Grapin-Botton et al., 1995). Hence, there is evidence that in embryonic development signals do spread through the neurectoderm to pattern it in a concentration-dependent manner.

iv. N-M gradient model

Another two-signal model was proposed by Saxén and Toivonen (1955, 1961). They induced mesoderm or neurectoderm using heterologous inducers that were implanted alone or together into a host. Alternatively, the inducers were separately administered to one population of cells to induce mesoderm and to another to induce neurectoderm. These cells were then dissociated and combined with each other in various ratios. The structures produced in both of these experimental approaches were different depending on whether the mesoderm and neurectoderm inducers, or the induced cell types, were used alone or in combination. These data led to the idea that there were two gradients: M, a caudo-cephalic gradient that induces mesoderm and caudalises neural structures and N, a shallow cephalo-caudal gradient that induces both anterior neural and placodal ectoderm. This is quite similar to the 'activation-transformation' model of Nieuwkoop et al. (1952, 1954) except for the induction of placodal ectoderm by N, which is not induced in the 'activation' step.

1.5.3 Vertical versus planar signalling and the difficulties with amphibians

The data reviewed in the experiments above strongly suggest that both vertical and planar signals are required for A-P patterning whereby planar signals from the organizer can spread along the neurectoderm to establish a crude A-P pattern which is subsequently refined by underlying mesoderm. Many of the experiments performed to investigate this issue have used amphibians although a mixture of species has been employed. This makes it rather difficult to compare the results. The response of these different species to the same treatments can generate varying results. For example, Barth (1941) discovered that, if removed, the presumptive neurectoderm of *Ambystoma punctatum* could undergo neural differentiation and

form anterior neural structures in the absence of dorsal mesoderm. This suggests that the dorsal mesoderm is not required for neural induction. However, Holtfreter (1945) repeated these experiments using *A. punctatum* and *Triton torosus* and found that, whilst 'autoneuralisation' did occur in the former, it did not occur in the latter. Also, Axolotl have been suggested to be particularly suitable to create exogastrulae as performed by Holtfreter because they are heavy, yolk-rich and a complete separation of endomesoderm and ectoderm takes place during the midblastula transition. However, in *Xenopus*, this separation does not occur before some mesoderm involution has already taken place that could explain how regional neural markers are generated in the ectoderm (Ruiz i Altaba, 1992). In *Triturus aplestris* there is a very strong tendency to involute, which impairs exogastrulation in a hypotonic medium (Chen et al., 2000). The difficulty with Axolotl in exogastrulae is that the bridge between the endomesoderm and ectoderm is very fine which could result in planar signals being kept below a threshold level required for planar signalling to induce and pattern the neurectoderm. Therefore, it is difficult to rule out vertical signalling in exogastrulae because exogastrulation could be incomplete (Holtfreter, 1933 on experiments by Goerttler, 1926 which suggested planar signals spreading in exogastrulae). Conversely, exogastrulation might prevent neural induction in the Axolotl (Holtfreter 1933) because it creates an unnaturally delicate region through which planar signals pass from dorsal mesoderm to presumptive neurectoderm (Chen et al., 2000). Even Keller sandwich experiments cannot rule out vertical signalling because invasion of the mesoderm-endoderm tissue at the leading edge of the mesodermal mantle can occur and could therefore provide vertical signals (Poznanski and Keller, 1997).

These problems cast a slight shadow on some of the conclusions reached regarding a role for only vertical or only planar signalling. They further the idea that, even when trying to analyse one method of signalling in isolation, elements of both planar and vertical signalling are probably required for A-P patterning.

1.5.4 An Updated Version of the 'Activation-Transformation' Model

The data suggesting that the AVE plays an important role in, and is required for, head development (Thomas and Beddington, 1996; Varlet, Collignon & Robertson, 1997; Shawlot et al., 1999; Dufort et al., 1998; Acampora et al., 1998; Rhinn et al.,

1998) and the recent characterisation of the avian hypoblast (Foley et al., 2000) suggest that an initial activation of the epiblast to a "pre-neural, pre-forebrain" state could be provided by these extra-embryonic tissues. When grafted into the chick area opaca, the hypoblast can transiently induce *Sox3*, *Otx2* (Foley et al., 2000) and *ERNI* (Streit et al., 2000) but not *Sox2* (Foley et al., 2000). Likewise, the AVE can only induce the initial phase of *Otx2* in the epiblast and further signals from the anterior mesendoderm are required to maintain its expression (Ang et al., 1994; Acampora et al., 1998; Rhinn et al., 1998) and whilst the AVE is required for head development it cannot induce anterior neural markers when grafted into a host epiblast (Tam and Steiner, 1999). To accommodate these data, Stern (2001) proposed a revised model of anterior-posterior patterning based on the 'activation-transformation' model. It proposes that the 'activation' step might not induce definitive anterior neural character but instead a pre-neural, pre-forebrain state and that the signals would originate from the AVE/hypoblast. These extra-embryonic endodermal tissues would then direct the movement of the prospective head. A second signal, perhaps from the node or PME, is then required to maintain the induced tissue and to give it a definitive anterior neural character. The 'transformation' signal would follow, emanating from the node. (Fig.1.4 from Stern, 2001). The experiments in this thesis were designed to test the three steps of this model.

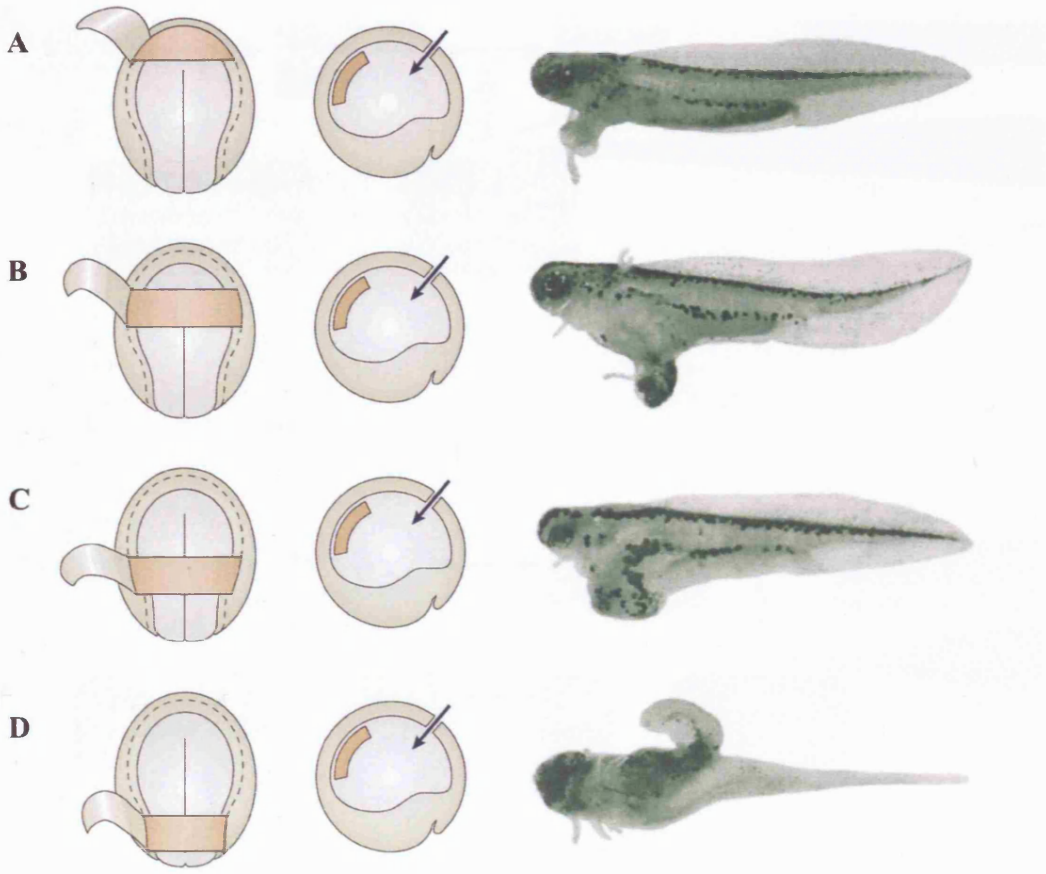


Figure 1.3 Mangold (1933) grafted regions of the invaginated archenteron roof into the blastocoele of a host embryo. Anterior portions of mesoderm generated ectopic rostral structures whilst progressively more posterior grafts resulted in increasingly caudal regions being produced. Taken from Stern, 2001.

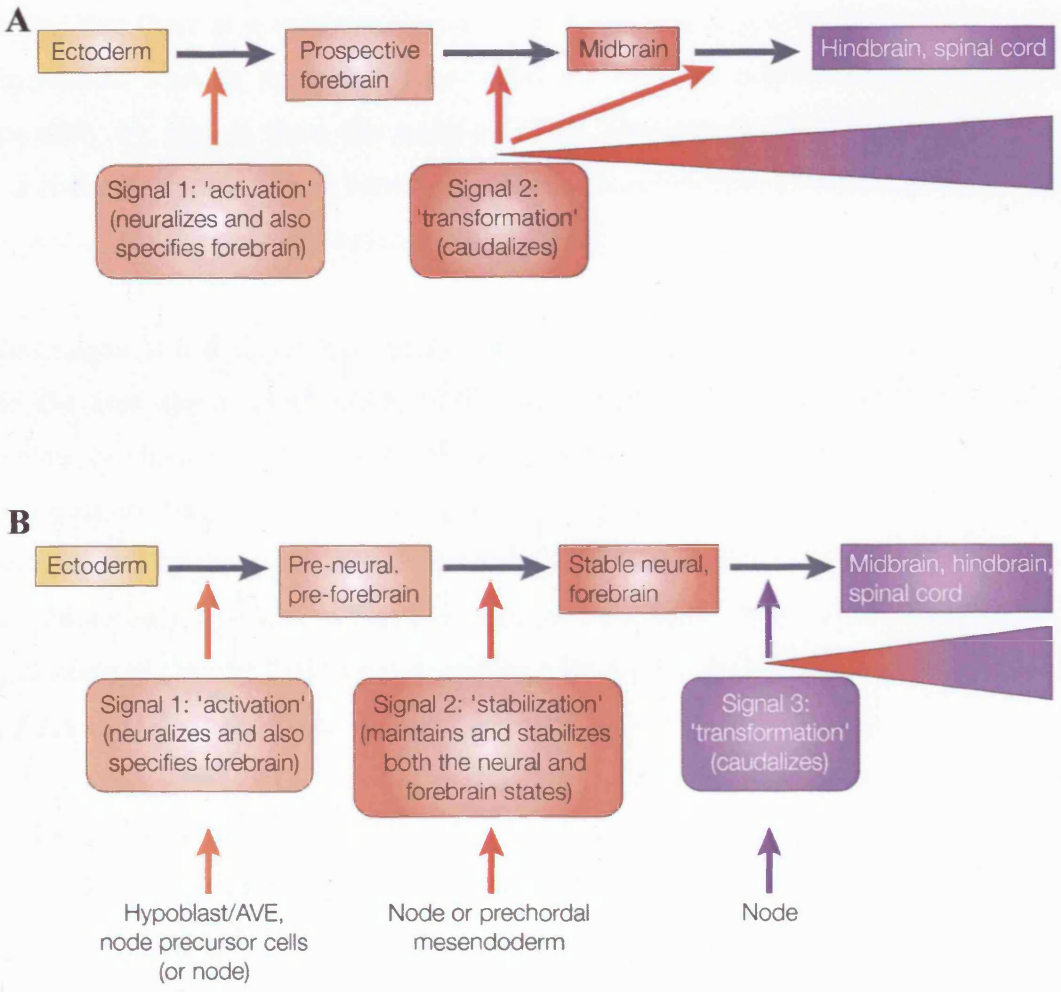


Figure 1.4 Representations of (A) Nieuwkoop and Nigtevecht's 1954 model of 'Activation-Transformation' and (B) the revised model (Stern, 2001). In A, the neur-ectoderm is induced and patterned by two signals the first of which 'activates' and results in a prospective forebrain stage and the second of which 'transforms' the axis into progressively more caudal structures. In B, the first step is sub-divided into an 'activation' step which induces a transient pre-neural, pre-forebrain state and a subsequent 'stabilisation' step that maintains the transient induction and creates a stable anterior neural state. Taken from Stern, 2001.

1.7 Aim of Thesis

The aim of this thesis is to look at anterior-posterior patterning of the neuraxis by testing the revised model of 'activation-transformation' (Stern, 2001). The model states that there is a transient activation of a pre-neural, pre-forebrain state by the hypoblast. This is maintained and stabilised to form anterior neural character, possibly by signals from the node or PME. The axis is then transformed by a caudalising gradient that is either of increasing time spent in the vicinity of a steady signal or an increasing strength of caudalising signal.

In Chapter 3, it is shown that the hypoblast is capable of inducing 4 epiblast markers in the area opaca: *Otx2*, *Sox3*, *ERNI* and *Cyp26A1*. *Sox3* and *ERNI* are induced within 1-2 hours of a hypoblast graft and *Otx2* and *Cyp26A1* appear after 3-4 hours. All markers disappear after 10-12 hours. The signalling properties of hypoblast were investigated by the application of RA and/or FGF and/or Wnt antagonists. RA is able to induce only *Cyp26A1* whilst FGF induces only *Sox3, ERNI* and *Otx2*. However, induction of *Otx2* by FGF is not consistent with that by the hypoblast. A combination of RA and FGF, rather than inducing all markers, results in a reduction in the level of *Otx2*, *Sox3*, *ERNI* and *Cyp26A1* induced. Wnt antagonists are unable to induce any of the genes analysed. Loss of function experiments show that RA is required for the hypoblast to induce *Cyp26A1* and that FGF is required for *Sox3* induction. Conversely, exogenous FGF in combination with a hypoblast graft down-regulates *Otx2* induction. In summary, the transient inducing abilities of the hypoblast can be attributed, in part, to FGF and RA signalling.

In Chapter 4, a combination of hypoblast grafts and BMP-, Wnt- and Nodal-antagonists are used to investigate whether the transiently induced markers are maintained. Inhibition of each signalling pathway, alone or together, in combination with the hypoblast, is able to maintain expression of *Sox3* and *ERNI*, complete with neural plate morphology but that mutual inhibition of *BMP* and *Wnt* is required for the maintenance of Otx2. *Cyp26A1* is never maintained by these factors and requires continued RA signalling for its maintenance. No combination of hypoblast plus inhibitors results in the induction of definitive neural marker, *Sox2*. This suggests

that maintaining the hypoblast-induced pre-neural, pre-forebrain state for 18 hours or more is not sufficient to induce neural character.

In Chapter 5, a series of node grafting experiments, placing a donor node of various stages in the prospective hindbrain region of a host, is performed to generate a secondary axis. Two types of secondary axis result: one growing in the plane of the host and one developing out of the plane. The former type is patterned equivalently to the host indicating a temporal gradient of signals patterning the ectopic axis or a re-specification of this axis by host tissue. The latter type of secondary axis develops according to the stage of the donor, indicating an intrinsic age and timing mechanism of the node. This suggested that node-derived presomitic mesoderm might have a role in patterning the axis that overrides patterning signals provided by the node.
To assess the role of the PSM, homotopic presomitic mesoderm transplantation experiments were performed. The PSM was replaced with that from an older or younger embryo or was removed altogether to look at the effect on neural tube patterning. The results show that older PSM has stronger caudalising effect on the neurectoderm and that PSM from younger donors anteriorises host neuraxis. However, a removal of PSM, or inhibition of Wnt signalling also anteriorises the neural tube, which suggests that it is indirect and results from a reduction in caudalising signal.

In conclusion, to compare these data with the modified 'activation-transformation' model (Stern, 2001), it would seem that there is a transient activation step that induces, pre-neural, pre-forebrain markers (also Foley et al., 2000; Streit et al., 2000) and involves the action of both RA and FGF. Also consistent with the model, the transient induction of these factors can be maintained by the addition of BMP- and/or Wnt-antagonists although a combination of both is required for the maintenance of *Otx2*. However, contrary to the model, this is not sufficient for neural induction, which suggests that a separate and distinct signal is required for this process. The transformation step requires signals from the posterior presomitic mesoderm although the mechnism is unclear.

Chapter 2: Materials & Methods

2.1 Embryos

Hen's eggs (Brown Bovan Gold) were obtained from Henry Stewart UK, Cambridge (0150760270) and quail eggs from Mr B.C. Potter, Cambridge (01487823084).

All embryos were staged according to Eyal-Giladi and Kochav (1976) for pre-streak stages using Roman numerals. Post-streak embryos were staged in Arabic numerals according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). See Figure 2.1.

Embryos were incubated for 2-48 hours at 38°C to acquire the correct stage for each experiment.

2.2 Basic Grafting Techniques

The chick embryo to be used as a host was transferred to a modified New culture (New, 1955; Stern & Ireland, 1981). The donor embryo was transferred to a dish containing Pannett-Compton (Pannett and Compton, 1924) and the region to be grafted excised using an insect pin attached to a pin vice.

When the graft was placed in the area opaca of the host, a region of yolky cells from the inner third of the anterior half of the area opaca was cleared from the ventral side. The graft was then placed in contact with the extra-embryonic ectoderm.

When the graft tissue was placed in the area pellucida, adjacent to the host node, a layer of endoderm was lifted, under which the graft was inserted, to allow the graft to be anchored in position

Following transplants, embryos were cultured for an additional 2-24 hours at 38°C.

2.3 Cells

COS-7 cells were transfected using Lipofectamine and Plus reagent (Invitrogen). Briefly, cells were plated at $1.5x10^6$ onto 35mm tissue culture dishes. The Lipofectamine-Plus-DNA were pre-incubated at room temperature: 1μg (1μg/μl) DNA was added to 100μl Optimem (GIBCO) with 6μl Plus reagent and left for 15 minutes. 4μl Lipofectamine reagent was added to 100μl Optimem and this was

added to the DNA solution and left for a further 15 minutes. This was then added to the cells, which had been transferred to 800µl serum-free DMEM (GIBCO) and incubated for 31/2 hours at 37°C. The medium was changed back to DMEM plus 10% calf serum and the cells were left overnight. The next day, the cells were counted and suspended at various concentrations, depending on the experiment. 20µl drops of the cell suspension were aliquoted onto the inside of the lid of a 6cm bacterial Petri dish. The base of the dish was filled with 5ml of PBS. The lid was carefully inverted and returned to its base. The cells were replaced in the incubator overnight during which time they aggregated into pellets.

Cell pellets were prepared for a day's experiments and were used both in Chapter 3 and Chapter 4 experiments. Therefore, although negative results were obtained for cell pellet grafts in Chapter 3, positive results were obtained for the same batches of pellets in Chapter 4.

Constructs used to transfect cells were:

Dkk1: chick coding region expression construct (E. Laufer)

Crescent: chick coding region, cloned into pMT23 (I. Skromne)

Chordin: chick coding region expression construct, cloned in pMT23 (A. Streit)

Xcer-S: *Xenopus* 'short' form, cloned in pCDNA 3.1: (E. De Robertis)

Cerberus: chick coding region expression construct in SLAX12 (M. Marvin)

2.4 In situ Hybridization

Embryos were assessed for expression of various markers by mRNA in situ hybridization using the protocol described by Stern (1998). Embryos were fixed at room temperature for 1-2 hours or at 4°C overnight in 4% paraformadelhyde (PFA) made up in phosphate buffered saline (PBS). They were subsequently transferred to 100% methanol and stored at -20°C.

To commence the protocol, embryos were rehydrated in methanol: PTW (PBS containing 0.1% Tween) series of 3:1, 1:1, 1:3 and finally washed 2x10 minutes PTW. A proteinase K step followed (concentration: 10µg/ml) for 15 minutes. They were rinsed in PTW and then post-fixed in 4% PFA with 0.1% gluteraldehyde for 25mins. Another two rinses in PTW followed and one in hybridization solution before being transferred to hybridization solution and placed in a 70°C water bath. Embryos were pre-hybridized for 4-6 hours before the Digoxigenin (Dig) or

fluorescein (fluor)-labelled mRNA probe was added. The probes were left to hybridize overnight at 70°C and the following morning embryos were washed twice in hybridization solution and once in a 1:1 mixture of hybridization solution and TBST (Tris-buffered saline plus 1% Tween-20), each for 30 minutes, at the same temperature. Embryos were removed from the water bath and rinsed 3 times and washed 3 times 1 hour in TBST at room temperature. They were then blocked for 3 hours in blocking buffer (TBST plus 5% heat inactivated goat serum plus 1mg/ml bovine serum albumin). Anti-Dig-AP or anti-fluor-AP antibodies (conjugated to alkaline phosphatase) were added at 1:5000 in blocking buffer and embryos were incubated overnight at 4°C (Roche). The next morning, embryos were returned to room temperature and washed 5 times 1hour in TBST. They were then washed 2 times 5 minutes in NTMT (0.1M NaCl, 1M Tris-HCL pH9.6, 0.05m MgCl2, 1% Tween-20) before being transferred into developing solution NBT/BCIP (Roche: 4.5µl of NBT stock: 75µg/ml in 70% Dimethylformamide [DMF] and 3.5µl BCIP stock: 50mg/ml in 100% DMF per 1.5ml) in NTMT until the colour reaction had developed (30 minutes – overnight). The embryos were then rinsed in PBS and post-fixed in 4% PFA before being stored at 4°C.

Probes used in the experiments were:

Otx2: chick Otx2, cloned pBS II; antisense probe: cut-Xho1, transcribe-T3 (L. Bally-Cuif)

Sox3: chick Sox3, cloned in pBSSK; antisense probe: cut-Pst1, transcribe-T7 (P. Scotting)

Sox2: chick Sox2, cloned pBSSK; antisense probe: cut-Pst1, transcribe-T7 (P.Scotting)

ERNI: chick ERNI, cloned pBS; antisense probe; cut-Kpn1, transcribe-T3 (A. Streit)

Cyp26A1: chick Cyp26A1, cloned in pBSSK; antisense probe: cut-BamH1, transcribe-T7 (E. Swindell)

Brachyury: chick brachyury, cloned in pBS; antisense probe: cut-Xba1, transcribe-T3 (V. Cunliffe)

Hoxb4: chick Hoxb9, cloned pBSK; antisense probe: cut-HindIII, transcribe-T3 (L. McNaughton)

Hoxb9: chick Hoxb9, cloned pBSK; antisense probe: cut-HindIII, transcribe-T3 (L. McNaughton)

2.5 Embedding and sectioning

Embryos were dehydrated in a gradient of ethanol:PBS of 1:3, 1:1, 3:1 and 100% ethanol. They were then transferred to toluene for 2 minutes and then to paraffin wax. Three changes of wax followed, at 65°C and then embryos were placed in moulds and cooled. The wax blocks were mounted for sectioning. Sections were cut at 8μm and mounted on glass slides. They were de-waxed in toluene and mounted with cover slips using Depex.

2.6 Photography

Photographs were taken using an Olympus SZH10 dissection microscope or a Vanox-T Olympus microscope and an Axiovision Leica digital camera. Composite images were prepared in Photoshop.

2.7 Data Analysis

When embryos were being analysed for the presence or absence of an induction, this was scored in the following ways. Embryos in which a strong induction was observed were analysed under the dissection microscope at high magnification and it was possible to determine from this that the expression of the induced marker resided in the area opaca and not in the ventrally placed hypoblast graft or cell pellet. A sample from each experiment was sectioned to ensure this was the case. Experiments that yielded host embryos with consistently faint inductions were analysed further by sectioning. If positively stained cells were observed in the responding tissue, then these would be considered weakly induced.

When an upregulation or downregulation of an induction was hypothesised, control experiments and mRNA detection were performed at the same time and when differences were observed between control and experimental group, this was noticeable by either a) an increase/decrease in the intensity of the staining but the region stained remained the same size and/or b) an equivalent intensity of staining but a greater/reduced size of stained region or c) an increase of staining or an absence of staining within an induced region, in the vicinity of a bead graft. When

these changes were observed and noted they were both unambiguous and occurring in the majority of embryos and were deduced by comparing photographs of wholemount embryos by eye as well as by sectioning a representative sample.

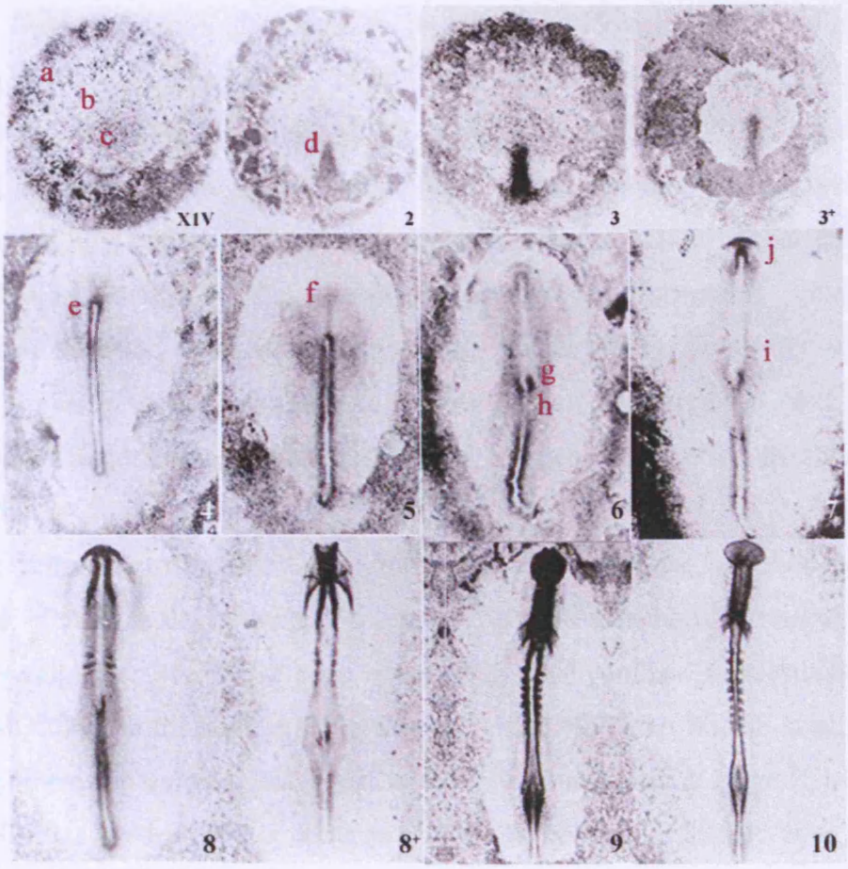


Figure 2.1 The stages of embryonic development in the chick (XIV-10). Modified from Hamburger and Hamilton, 1951. Stages described in detail in Chapter 1.2.

a. area opaca; b. epiblast; c.hypoblast; d. primitive streak; e. node; f. PME and head process; g. first somite forming; h. presomitive mesoderm; i: first somite; j: neural folds visible in head region.

Chapter 3: Characterisation of 'activation' signals from the hypoblast

3.1 Introduction

The hypoblast and its murine equivalent, the AVE, have been implicated in the initial stages of head formation (Thomas & Beddington, 1996; Varlet, Collignon & Robertson, 1997Acampora et al., 1998; Dufort et al.,1998; Rhinn et al., 1998; Shawlot et al., 1999; Foley et al., 2000; Liguori et al., 2003). The hypoblast is capable of inducing the pre-neural markers *Sox3*, *Otx2* and *ERNI* in the area opaca but this induction is transient (Foley et al., 2000; Streit et al., 2000). These data led to a modification of the Nieuwkoop "activation–transformation" model to accommodate an early, transient induction state (Stern, 2001). This early activation step was proposed to induce a transient, "pre-neural, pre-forebrain state" in the epiblast which requires later stabilising signals to become specified as both neural and forebrain.

The exact timing for the inducing abilities of the hypoblast has not yet been established. Neither is the molecular nature of this induction known. However there are some candidates: *FGF8* has been detected in the hypoblast at prestreak stages (Streit et al., 2000) and *FGF3* in the pre-streak area pellucida (Wilson et al., 2000). The RA synthesising enzyme, *RALDH2,* although not detected at stage XII (Wilson et al., 2000), has been observed at stage 3 in the hypoblast (Halilagic et al., 2003). Several Wnt antagonists including *Dkk1*, *crescent* and *Cerberus* are also expressed in the hypoblast of the pre-streak embryo (Pfeffer 1997; Zhu et al., 1999; Foley et al., 2000; Skromne & Stern 2001, Chapman et al., 2002). Therefore three signalling pathways are good candidates for the activating properties of the hypoblast: FGF, RA and Wnt. Perhaps paradoxically, all three of these families of secreted factors have been implicated in the subsequent caudalisation (transformation) step as well as mesoderm induction (FGF: Cox et al., 1995; Pownall et al., 1996; Muhr et al., 1997; 1999; Hardcastle et al., 2000; Koshida et al., 2002; Rentzsch et al., 2004 etc., RA: Blumberg et al., 1997; Chen et al., 2001; Dupe and Lumsden, 2001; Kaiser et al., 2003; Shiotsugu et al., 2004; Molotkova et al., 2005 etc., Wnt: McGrew et al., 1997; Domingos et al., 2001; Kiecker and Niehrs, 2001; Kudoh et al., 2002; Nordstrom et al., 2002; Shimizu et al., 2005) and will be discussed in Chapter 5. However, FGF (Wilson et al., 2000; Pera et al., 2003), RA (Knezevic and Mackem, 2001; Shiotsugu

et al., 2004), and both Wnts (Sokol et al., 1995; Baker et al., 1999; Liu et al., 1999; Wessely 2001) and Wnt antagonists (Glinka et al., 1997;Wilson et al., 2000; Nambiar et al., 2004) have also been implicated in the initial steps of head formation and neural induction although their role in the hypoblast has never been investigated. This chapter investigates the signals that might underlie the ability of the hypoblast to induce expression of four early 'pre-neural/pre-forebrain' markers: *Sox3*, *ERNI*, *Otx2* and *Cyp26A1*. *Sox3* is a pre-neural marker in the chick and at prestreak stages it is expressed throughout the epiblast and later becomes restricted to the neural plate (Rex et al., 1997; Streit et al., 1998; 2000). *ERNI* is one of the first genes to be induced following a graft of Hensen's node (Streit et al., 2000). During normal development, it is initially expressed throughout the epiblast at pre-streak stages and then becomes downregulated in the neural plate as *Sox2* starts to be upregulated at stage 4+. *Otx2* has three phases of expression (Bally-Cuif et al., 1995). Initially it is also expressed in the epiblast prior to streak formation; during gastrulation (stages 3-4) it appears briefly in the tip of the streak and node. It then becomes restricted to the prospective forebrain, midbrain and underlying mesendoderm region from about stage 4+ onwards (Bally-Cuif et al., 1995). *Cyp26A1* is induced in response to RA (Martinez-Ceballos et al., 2001; Dobbs-McAuliffe et al., 2004), which it subsequently degrades (Sonneveld et al., 1999). It too is expressed in the epiblast at pre-streak stages (personal observations) and then follows the expression of Otx2, briefly appearing in the node and then just after gastrulation it becomes restricted to the anterior epiblast of the area pellucida (Swindell et al., 1999; Blentic et al., 2003). This chapter also assesses the dynamics of induction of these markers. The stability of the induction is examined by determining for how long they are expressed following a graft. Finally, the role of FGF, RA and Wnt antagonists in this process are investigated by performing gain and loss of function experiments. The results indicate that all markers are induced by the hypoblast, but not simultaneously: *Sox3* and *ERNI* are induced first, and this induction lasts for 10-12 hours. FGF can induce *Sox3*, *ERNI* and *Otx2* whilst *Cyp26A1* is induced by RA. No markers are induced by Wnt antagonists. I propose an early role for both FGF and RA signalling by the hypoblast, and that these signals are subsequently antagonised to allow for the induction of pre-forebrain markers, or to protect the pre-forebrain state from transforming influences.

et al., 2004), and both Wnts (Sokol et al., 1995; Baker et al., 1999; Liu et al., 1999; Wessely 2001) and Wnt antagonists (Glinka et al., 1997;Wilson et al., 2000; Nambiar et al., 2004) have also been implicated in the initial steps of head formation and neural induction although their role in the hypoblast has never been investigated. This chapter investigates the signals that might underlie the ability of the hypoblast to induce expression of four early 'pre-neural/pre-forebrain' markers: *Sox3*, *ERNI*, *Otx2* and *Cyp26A1*. *Sox3* is a pre-neural marker in the chick and at prestreak stages it is expressed throughout the epiblast and later becomes restricted to the neural plate (Rex et al., 1997; Streit et al., 1998; 2000). *ERNI* is one of the first genes to be induced following a graft of Hensen's node (Streit et al., 2000). During normal development, it is initially expressed throughout the epiblast at pre-streak stages and then becomes downregulated in the neural plate as *Sox2* starts to be upregulated at stage 4+. *Otx2* has three phases of expression (Bally-Cuif et al., 1995). Initially it is also expressed in the epiblast prior to streak formation; during gastrulation (stages 3-4) it appears briefly in the tip of the streak and node. It then becomes restricted to the prospective forebrain, midbrain and underlying mesendoderm region from about stage 4+ onwards (Bally-Cuif et al., 1995). *Cyp26A1* is induced in response to RA (Martinez-Ceballos et al., 2001; Dobbs-McAuliffe et al., 2004), which it subsequently degrades (Sonneveld et al., 1999). It too is expressed in the epiblast at pre-streak stages (personal observations) and then follows the expression of Otx2, briefly appearing in the node and then just after gastrulation it becomes restricted to the anterior epiblast of the area pellucida (Swindell et al., 1999; Blentic et al., 2003). This chapter also assesses the dynamics of induction of these markers. The stability of the induction is examined by determining for how long they are expressed following a graft. Finally, the role of FGF, RA and Wnt antagonists in this process are investigated by performing gain and loss of function experiments. The results indicate that all markers are induced by the hypoblast, but not simultaneously: *Sox3* and *ERNI* are induced first, and this induction lasts for 10-12 hours. FGF can induce *Sox3*, *ERNI* and *Otx2* whilst *Cyp26A1* is induced by RA. No markers are induced by Wnt antagonists. I propose an early role for both FGF and RA signalling by the hypoblast, and that these signals are subsequently antagonised to allow for the induction of pre-forebrain markers, or to protect the pre-forebrain state from transforming influences.

3.2 Materials & Methods

3.2.1 Grafting

Donor embryos were incubated for 2-7 hours, depending on the time of year, to obtain stages between XII and XIII. They were transferred into Tyrode's saline and the central portion of the hypoblast was removed with an insect pin.

Host embryos were incubated until stage 3 and transferred to modified New culture (Stern and Ireland, 1981). Grafts were placed according to the diagram below, in position 'X' of the host embryo. The hypoblast graft was placed in this region: the inner third-half in the area opaca at a level anterior to that of the host node. The cultures were grown for a further 1-18 hours.

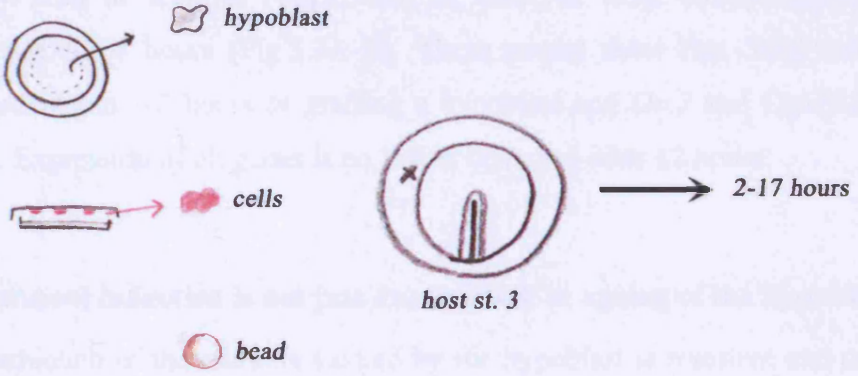
For FGF bead grafts, lyophilised proteins were reconstituted in PBS with 1% BSA and stored at -80oC at 100μg/ml. Heparin-coated acrylic beads (Sigma) were soaked in either FGF4 or FGF8 (Sigma) at various concentrations (FGF4: 5μ/ml, 50μg/ml; FGF8: 50μg/ml, 100μg/ml). Briefly, heparin beads were rinsed three times in PBS before being resuspended in 5μl of PBS. 5μl of FGF solution were added and the beads incubated at 4°C for 1 hour or until required (whichever was longer). The beads were then emptied into a Petri dish containing PBS and kept on ice during the grafting experiments. Control beads were prepared in the same way but using PBS alone.

For other bead grafts, all-*trans* RA (Sigma) and SU5402 (Calbiochem) were dissolved in DMSO (dimethylsulphoxide) at 10mg/ml and 1mM respectively and stored in aliquots at -80°C. Citral (Sigma) was already in solution and was made up to $1x10^{-4}$M in DMSO prior to use. They were bonded to AG1X2 beads at various concentrations (RA: 0.001mg/ml, 0.01mg/ml, 0.1mg/ml, 0.5mg/ml, 1mg/ml; SU5402 25mM, 250mM, 1mM). Briefly, AG1X2 beads were rinsed three times in PBS and then resuspended in 100% DMSO. They were rinsed again in DMSO and then resuspended in a small volume before the appropriate factor was added. The beads were incubated in the dark for at least one hour. They were then rinsed twice in DMSO and then three times in PBS before being transferred to a Petri dish containing PBS and kept in the dark during experiments. Control beads were prepared the same way but using only DMSO and PBS.

Beads were grafted into the area opaca, in the same position as other grafts, either alone or in combination with a hypoblast and embryos were incubated in culture for 2-6 hours.

For SU5402 experiments, hypoblasts were occasionally pre-incubated in 25nM SU5402 prior to grafting. Hypoblasts were incubated in this solution for 30 minutes in the dark before being grafted into a host together with an SU5402 bead.

For cell grafting experiments, cells were transfected as in Chapter 2) with constructs expressing either *Dkk1* or *crescent* and cell pellets were produced to contain approximately 1000 cells.



3.3 Results

3.3.1 The Hypoblast Transiently Induces Pre-Neural Genes in the Area Opaca

i. A time course for induction by the hypoblast

It has been shown that hypoblast grafts can transiently induce the expression of *Otx2*, *Sox3* and *ERNI* in the area opaca when grafted in a stage 3+ to 4 host embryo (Foley et al., 2000; Streit et al., 2000). It was reported that *Otx2* is induced after 4-6 hours whereas *Sox3* does not appear until 8 hours following the graft, and *ERNI* is visible after 3 hours. When the host embryos were incubated for 18-20 hours no expression of *Sox2*, *Sox3* or *Otx2* could be detected.

Here, a time-course has been conducted for the inductive properties of the hypoblast using *Sox3*, *Otx2*, *ERNI* as well as *Cyp26A1*. Host embryos were at stage 3 rather than 3+/4, because these younger embryos appeared to be more responsive to the hypoblast signals.

When embryos are incubated for 1-2 hours following a hypoblast graft, there is some induction of *ERNI* (6/8) and *Sox3* (5/8) (Fig.3.1 A,B, E,F) but there is no expression of *Otx2* (0/10) or *Cyp26A1* (0/9) (Fig.3.1 C,D,G,H). *Otx2* (8/9) and *Cyp26* (6/8) appear later, after 3-4 hours (Fig. 3.1 K,L). The expression of these genes remains in the area opaca for 10 hours (*Sox3*: 3/5; *ERNI*: 4/7; *Otx2*: 2/6; *Cyp26A1*: 1/7), (Fig.3.2 E-H) but none of them can be detected 12 hours after grafting (*Sox3*: 0/6; *ERNI*: 0/6, *Otx2*: 0/6, *Cyp26A1*: 0/6) (Fig.3.2 I-L). There is no reappearance of expression between 12 and 17 hours post-graft (*Sox3*: 0/10; *ERNI*: 0/11; *Otx2*: 0/12; *Cyp26A1*: 0/12) (Fig3.2 M-T). The induction by the hypoblast is not due to hypoblast cells contributing to the ectoderm (Foley et al, 2000) and the ectodermal expression can be clearly seen in sections (Fig.3.3a-d) of embryos from embryos grafted with a hypoblast for 6 hours (Fig.3.3A-D). These results show that *Sox3* and *ERNI* are induced within 1-2 hours of grafting a hypoblast and *Otx2* and *Cyp26A1* after 3-4 hours. Expression of all genes is no longer observed after 12 hours.

ii. Transient induction is not just due to death or ageing of the hypoblast.

The induction of the markers studied by the hypoblast is transient and one possible reason for the loss of induced gene expression after 12 hours is that the grafted hypoblast cells do not survive. It is also possible that as the grafted hypoblast ages, it no longer emits the inducing signals, which may be required continuously to maintain the induction. To eliminate both possibilities, a hypoblast was implanted and the embryos incubated for 8 hours. A second, freshly-dissected hypoblast was then grafted on top of the original graft. The embryos were incubated for a further 8 hours and analysed. No expression was observed of *Sox3* (0/6), *ERNI* (0/6), *Otx2* (0/8) or *Cyp26A1* (0/8). This suggests that the transitory nature of the induction of these genes by the hypoblast grafts is not due to death of the graft or to loss of its inducing ability as it ages.

3.3.2 Early markers are induced by FGF and RA but not by Wnt antagonists.

i. FGF4 & FGF8 induce *Sox3, ERNI* and *Otx2*

FGF8 is expressed in the hypoblast at early stages of development (Streit et al., 2000) and it might therefore play a role in the induction of *Sox3*, *ERNI*, *Otx2* and *Cyp26A1*. Indeed, FGFs have been shown to induce both *Sox3* and *ERNI* previously (Streit et al., 2000). The following results are summarised in a table below. A graft of an FGF4- or FGF8- (each at 50μg/ml) soaked bead grafted in the area opaca for 6 hours induced *Sox3* (9/10), *ERNI* (5/5), *Otx2* (10/25) but no *Cyp26A1* (0/10) (Fig.3.3E-H). However, at this concentration and timing of FGFs, *brachyury* was also induced (4/4) (Fig.3.4 B). Not only is *brachyury* not induced by the hypoblast (0/4) (Fig.3.4 A) but a grafted hypoblast can prevent the induction of *brachyury* above it (although not beyond the graft) by 100μg/ml FGF8 (4/4)(Fig. 3.4 C) or 50μg/ml FGF4 (4/4) and can also down-regulate the endogenous expression of *brachyury* in the mesoderm emerging from the primitive streak (4/5) (Fig.3.4 D). To avoid the possibility that the *brachyury*-expressing cells (presumably mesendoderm) is responsible for inducing the genes (rather than the hypoblast directly), different concentrations of FGF were tested for their ability to induce the marker genes and *brachyury*. Beads of different concentration of either FGF8 or FGF4 were placed into the area opaca of stage 3 host embryos, which were then incubated for varying times. The expression of *brachyury*, *Sox3* and *ERNI* were analysed to find a concentration of FGF that induced *Sox3* and *ERNI* without also inducing *brachyury*. After two hours' incubation with either FGF4- (5 or 50μg/ml) or FGF8- (50 or 100μg/ml) soaked beads, *Sox3* was weakly induced by 50μg/ml FGF4 (4/8) (Fig.3.5 D) and 100μg/ml FGF8 (5/8) (Fig.3.5 J) and *ERNI* was induced at low levels by 5μg/ml FGF4 (3/4) and 50μg/ml of FGF8 (4/4) (Fig.3.5 B,H) and it was induced strongly by 50μg/ml FGF4 (4/4) and 100μg/ml FGF8 (4/4) (Fig.3.5 E,K) whilst there was no induced expression of *brachyury* (0/4 for each concentration of FGF)(Fig. 3.5 C,F,I,L). *Otx2* was induced by both 50 μg/ml FGF4 (7/18) and 100μ/ml FGF8 (3/7) but only after 5-6 hours and at the higher concentrations of FGF, which also induced *brachyury*. The expression of *Otx2* induced by FGF appeared as a crescent shape pointing towards the embryo proper and did not contact the bead (Fig.3.3 G). The shape of the induced domain raises the possibility that other factors may cooperate

with FGF for the induction of *Otx2*. To test whether these hypothetical factors are also produced by the hypoblast (rather than by an adjacent area of the host embryo), a bead of FGF8 (100μg/ml: n=10) or FGF4 (50μg/ml: n=10) was grafted in the area opaca, as before, near the boundary with the area pellucida; on the other side of the bead, near the outside edge of the area opaca, an ectopic hypoblast was grafted. The aim of this experiment was to identify if some factor in the grafted hypoblast could extend the crescent-shaped expression of *Otx2* induced by FGF into a complete ring. The results show that whilst there is some connection between the *Otx2* induced by the FGF and that induced by the grafted hypoblast, a complete ring does not form around the bead (0/20)(* in Fig.3.4 M,N). Therefore, factors involved in the induction of *Otx2* by FGF are not present in the hypoblast but might exist in the anterior epiblast.

Since in these experiments FGF beads induce *brachyury* as well as *Otx2* it is important to determine whether any induced mesoderm could inhibit the induction of *Otx2* near the bead. To look at this, FGF beads were grafted along with cell pellets expressing a Nodal-specific inhibitor, Cerberus-short (XCerS) (Piccolo et al., 1999) which inhibits the induction of *brachyury* by 100μg/ml FGF8 (4/4). This combination decreased the expression of *Otx2* compared to the control side (FGF and mock-transfected cells) (4/5)(Fig.3.4 O). These data suggest that FGF might induce *Sox3* and *ERNI* directly. *Otx2* induction by the hypoblast would appear not to be mimicked by FGF but instead FGF might be indirectly inducing a later phase of *Otx2* expression via mesendoderm, and for this induction another unknown signal is required, possibly from the anterior epiblast of the embryo proper. The signal produced by the hypoblast to induce *Otx2* is therefore unclear.

Summary of markers induced by FGF4 and FGF8:

	FGF4 5ugl/ml	FGF4 50ug/ml	FGF8 50ug/ml	FGF 100ug/ml
2 hours	ERNI + Sox3 – Otx2 -Bra -	ERNI ++ Sox3 + Otx2 - Bra -	ERNI + Sox3 – Otx2 – Bra -	ERNI ++ Sox3 + Otx2 – Bra -
6 hours	N/A	ERNI +++ Sox3 +++ Otx2 ++ Bra +++ Cyp26A1 -	ERNI ++ Sox3 ++ Otx2 – Bra ++ Cyp26A1 -	ERNI +++ Sox3 +++ Otx2 ++ Bra +++ Cyp26A1 -

ii. Retinoic Acid can induce *Cyp26A1*.

The hypoblast has been shown to express *RALDH2* at stage 3 (Halilagic et al., 2003). RA is therefore a candidate molecule for the inducing abilities of the hypoblast. *Cyp26A1* is induced by hypoblast grafts but not by FGF (see above). Experiments to uncover whether RA can induce *Cyp26A1* were performed by grafting 0.001-0.1mg/ml RA-soaked beads in the area opaca of a stage 3 embryo and incubating for 1-5 hours (Fig.3.6). As predicted, *Cyp26A1* was induced (4/4 for each concentration and time point). However, neither *Sox3, ERNI* nor *Otx2* were induced by 0.01 or 0.1 mg/ml RA after 5 hours (*Sox3*: 0/8; *ERNI*: 0/8, *Otx2*: 0/10) (Fig.3.3I-K). Therefore, FGFs are able to induce *Sox3*, *ERNI* and *Otx2* whilst RA can induce *Cyp26A1* but not the other markers.

iii. FGF & Retinoic Acid antagonise each others' ability to induce *Sox3*, *ERNI*, *Otx2* and *Cyp26A1*

The above results show that between FGF and RA, all of the markers under analysis are induced. This suggests that a combination of RA and FGF might induce the full range of markers as does a hypoblast graft. To test this a bead of FGF and a bead of RA were placed together in the area opaca of a stage 3 embryo that was then incubated for 4-6 hours. Surprisingly, rather than enhancing the induction, all markers are induced less robustly by the combination than by either of the factors alone. The addition of RA to FGF decreased the induction of *Sox3* (8/10), *ERNI* (6/7) and *Otx2* (10/10) compared to their induction by FGF alone (Fig.3.3M-O) as judged by a smaller region of the area opaca expressing the marker per embryo (compare E with M, F with N, G with O). *Cyp26A1* expression, which is strongly induced by RA, is lost at the point of contact with the FGF bead (6/10) (Fig.3.3 P). This suggests that RA and FGF are not acting in a simple combinatorial manner to induce these four markers.

iv. Wnt antagonism cannot induce *Sox3*, *ERNI*, *Otx2* or *Cyp26A1*

Since neither RA or FGF nor the two combined can induce the full range of markers induced by the hypoblast, another signalling pathway, Wnt, was tested. Wnt antagonists are expressed by the hypoblast (Pfeffer 1997; Zhu et al., 1999; Foley et al., 2000; Skromne & Stern 2001, Chapman et al., 2002) and it has been shown that Wnt must be antagonised for FGF to induce neural tissue (Wilson et al., 2001) so the ability of Wnt antagonists Dkk1 and crescent to induce *Sox3* and *ERNI* was tested by grafting cell pellets secreting these two factors into the area opaca of stage 3 embryos which were then incubated for 6 hours. These factors did not induce *Sox3* (0/10) or *ERNI* (0/10) (*Otx2* and *Cyp26A1* were not tested). Wnt antagonists were also grafted with beads coated in 50µg/ml FGF8 (which elicits a low-level induction of *Sox3* and a stronger induction of *ERNI*) and incubated for 2 hours to see whether the Wnt antagonists could enhance the induction by FGF8. However, no difference was observed as compared to FGF alone (*Sox3*: n=10; *ERNI*: n=9).

Whilst FGF does seem to induce *Sox3* or *ERNI* directly, it appears to induce *Otx2* only via mesoderm and might even inhibit the ability of the hypoblast to induce *Otx2* (see above). Therefore, other factors in the hypoblast might act to induce *Otx2*. RA cannot do this alone and therefore it was investigated whether a combination of RA and Wnt antagonists in the area opaca could induce *Otx2*. Pellets of *Dkk1*- and *crescent*-expressing cells were grafted alongside a bead of RA in the area opaca and host embryos incubated for 4-6 hours. No induction of *Otx2* was observed (0/6). Whilst these experiments using Wnt antagonists are by no means extensive, they do not reveal a role for Wnt antagonism in the transient inductions by the hypoblast.

Wnt itself might be required at this stage since it is necessary for dorsalisation and further experiments to test this should be performed.

3.3.3 FGF, RA and their antagonists in combination with the Hypoblast

i. The hypoblast requires FGF signalling to induce Sox3 but exogenous FGF inhibits the induction of Otx2.

The finding that FGFs induce *brachyury* as well as *Sox3*, *ERNI* and *Otx2* raises the possibility that the induction of the latter makers may be indirect. FGFs also reduce the ability of RA to up-regulate *Cyp26A1*. To test whether FGF is required by the hypoblast to induce these factors, hypoblasts were grafted together with beads coated

in 250mM SU5402. Prior to grafting, hypoblasts were pre-incubated in 25mM SU5402. In the control experiment, a hypoblast was grafted with a bead soaked in PBS. The embryos were incubated for 6 hours. SU5402 did not reduce the induction of *ERNI* (0/8) or *Cyp26A1* (0/10) (Fig.3.4 F,H). However, *Sox3* induction was reduced by inhibiting FGF signalling as judged by a lower level of expression surrounding the SU5402 bead (8/14) (Fig.3.4 E). Conversely, *Otx2* induction was increased when SU5402 was added to the hypoblast graft, detected by an increase in expression around the bead (10/16) (Fig.3.4 G). Therefore, *Sox3* induction by the hypoblast requires FGF signalling whilst *Otx2* is induced more robustly when FGF signalling is inhibited.

The loss of FGF signalling produces changes in the induction by the hypoblast of *Sox3* and *Otx2*. To test whether exogenous FGF, applied with a hypoblast has an effect on the induction of the markers, beads soaked in 50µg/ml FGF8 were grafted into a host embryo alongside a hypoblast graft and incubated for 6 hours. *brachyury* expression following these grafts was checked to ensure that mesoderm was not induced; it was not expressed in the region of the hypoblast graft (0/5) (Fig.3.4 C). The combination of FGF8 and hypoblast graft resulted in no change in the induction of *Sox3* (0/4) or *ERNI* (0/4). Surprisingly, although RA-mediated induction of *Cyp26A1* was reduced upon addition of FGF (see above), there was no change when FGF was added to the hypoblast graft (0/6)(Fig.3.4 L). However, there was a reduction in the induction of *Otx2* (7/10) (Fig.3.4 K).

These data suggest that exogenous FGF signalling inhibits *Otx2* induction by the hypoblast but that the hypoblast can prevent exogenous FGF from blocking *Cyp26A1* expression, indicating that *Otx2* and *Cyp26A1* are repressed by FGF via two different mechanisms or that the two markers are sensitive to different amounts of FGF signalling.

ii. RA signalling is required in the Hypoblast for *Cyp26A1* induction

Both the hypoblast and RA-beads induce *Cyp26A1*. To investigate whether the hypoblast induces *Cyp26A1* through RA, the RA inhibitor citral was used. Citral is an inhibitor of RALDH2, the final enzyme in the synthesis pathway of RA (Kikonyogo et al., 1999: Berggren et al., 2001). Citral was applied to beads at a concentration of $1x10^{-4}$M. Beads were grafted into the area opaca of stage 3 hosts

with a hypoblast graft. The control side of the embryo was grafted with a hypoblast and a PBS bead. Embryos were incubated for 6 hours. The expression of *Cyp26A1* was down-regulated or abolished on the side with the citral bead (8/10)(Fig.3.4 P). This suggests that RA synthesis is required for induction of *Cyp26A1*.

The addition of RA to an FGF bead graft reduces the induction of *Sox3* and *ERNI* (Fig 3.3 M,N). To test whether exogenous RA affects the induction of these two genes, a bead soaked in 0.01 or 0.1mg/ml RA was added to a hypoblast graft. Both are still induced although the induction by the hypoblast of *Sox3* is somewhat reduced upon the addition of RA as determined by the size of the induced area (0.01mg/ml RA: *Sox3*: 3/8; *ERNI*: n=9; 0.1mg/ml *RA*: Sox3: 5/8; *ERNI*: n=8) (Fig.3.4 I,J and compare the size of the induced domain of *Sox3* in Fig.3.4 I with Fig.3.3 A and of *ERNI* in Fig.3.4 J with Fig.3.3 B). This suggests either that the excess RA inhibits the ability of the hypoblast to induce these *Sox3*, or that its expression is regulated after induction by RA.

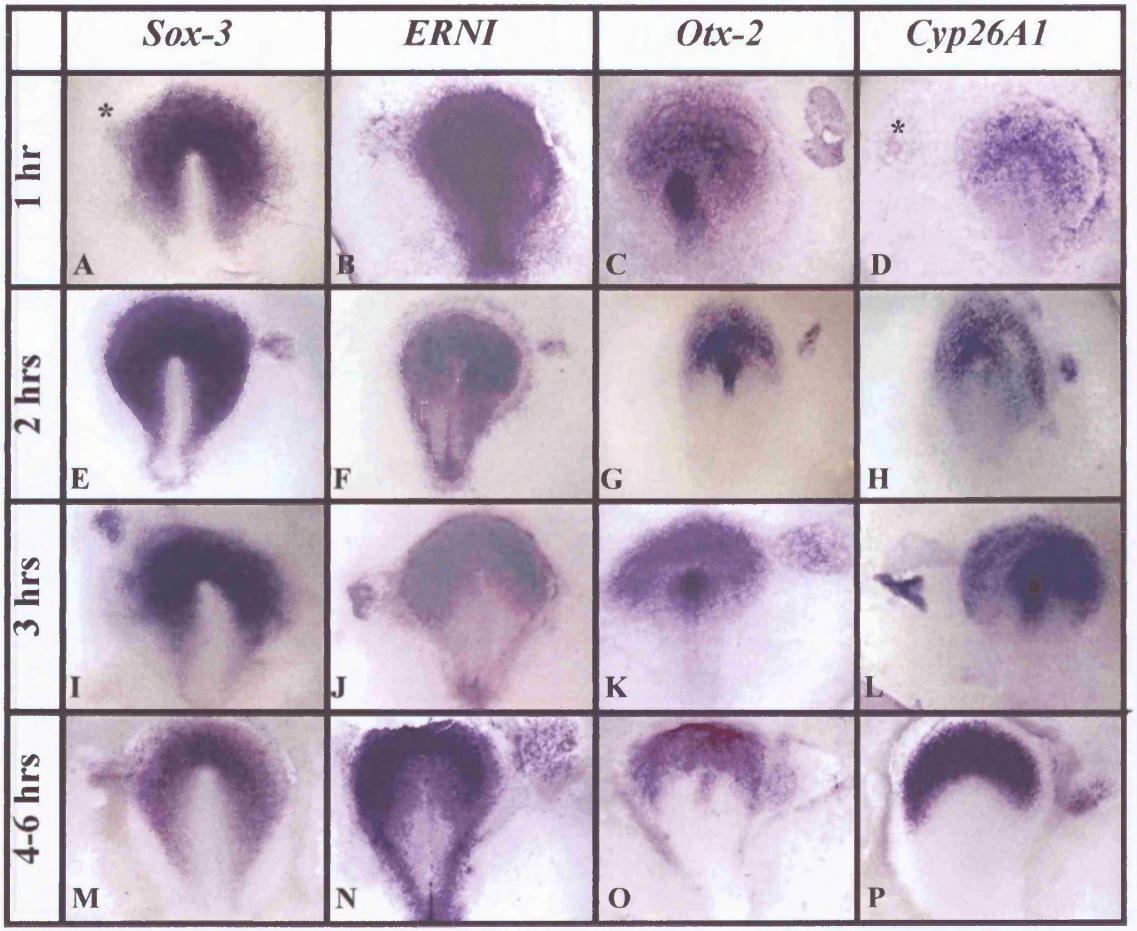


Figure 3.1 The hypoblast induces *Sox3* and *ERNI* within 1-2 hours and *Otx2* and *Cyp26A1* within 3-4 hours. * indicates position of hypoblast graft when little/no expression is seen.

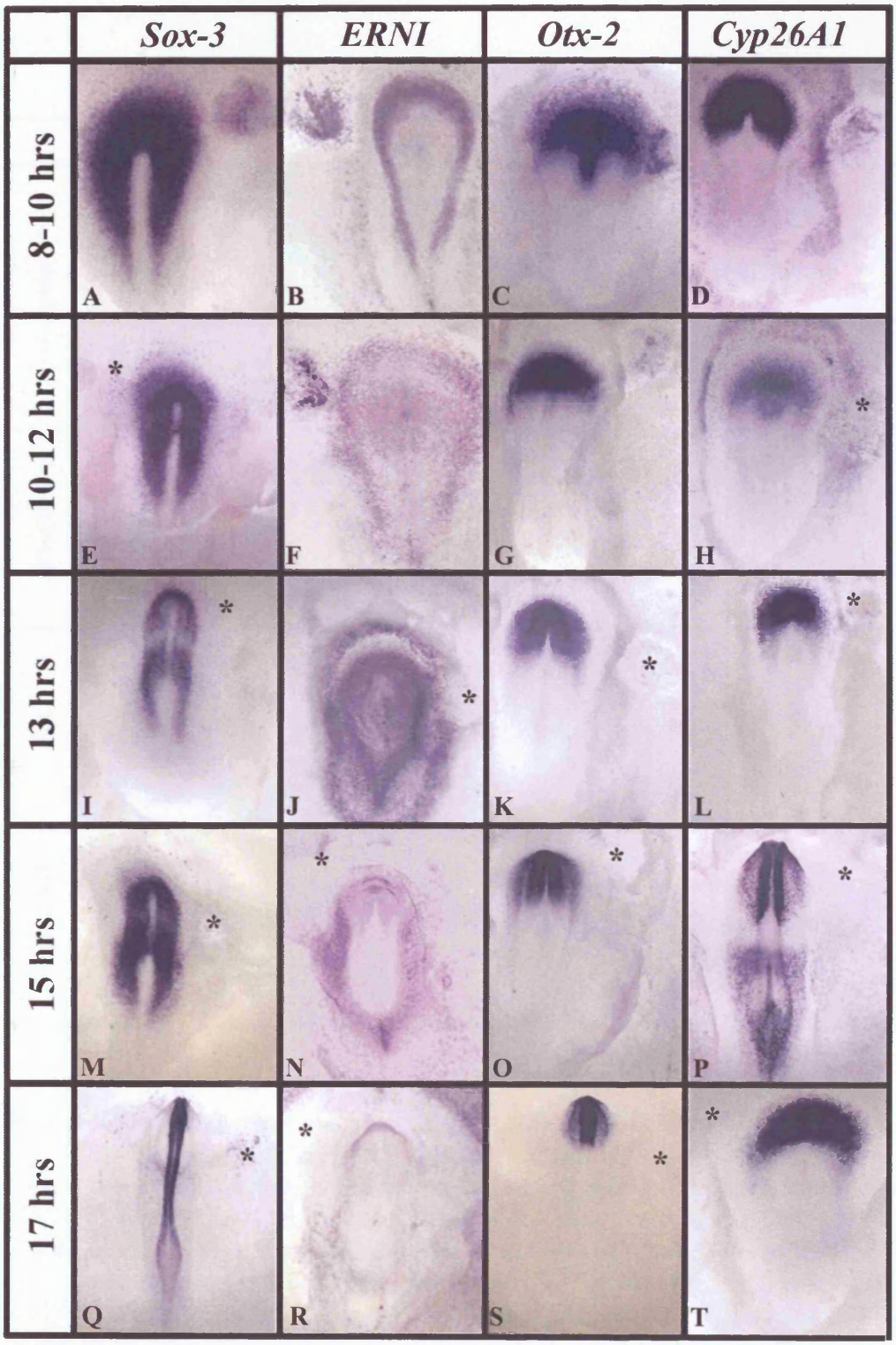


Figure 3.2 Hypobast-mediated induction of *Sox3, ERNI, Otx2* and *Cyp26A1* is lost after 10-12 hrs. * indicates position of graft when little/no expression is observed.

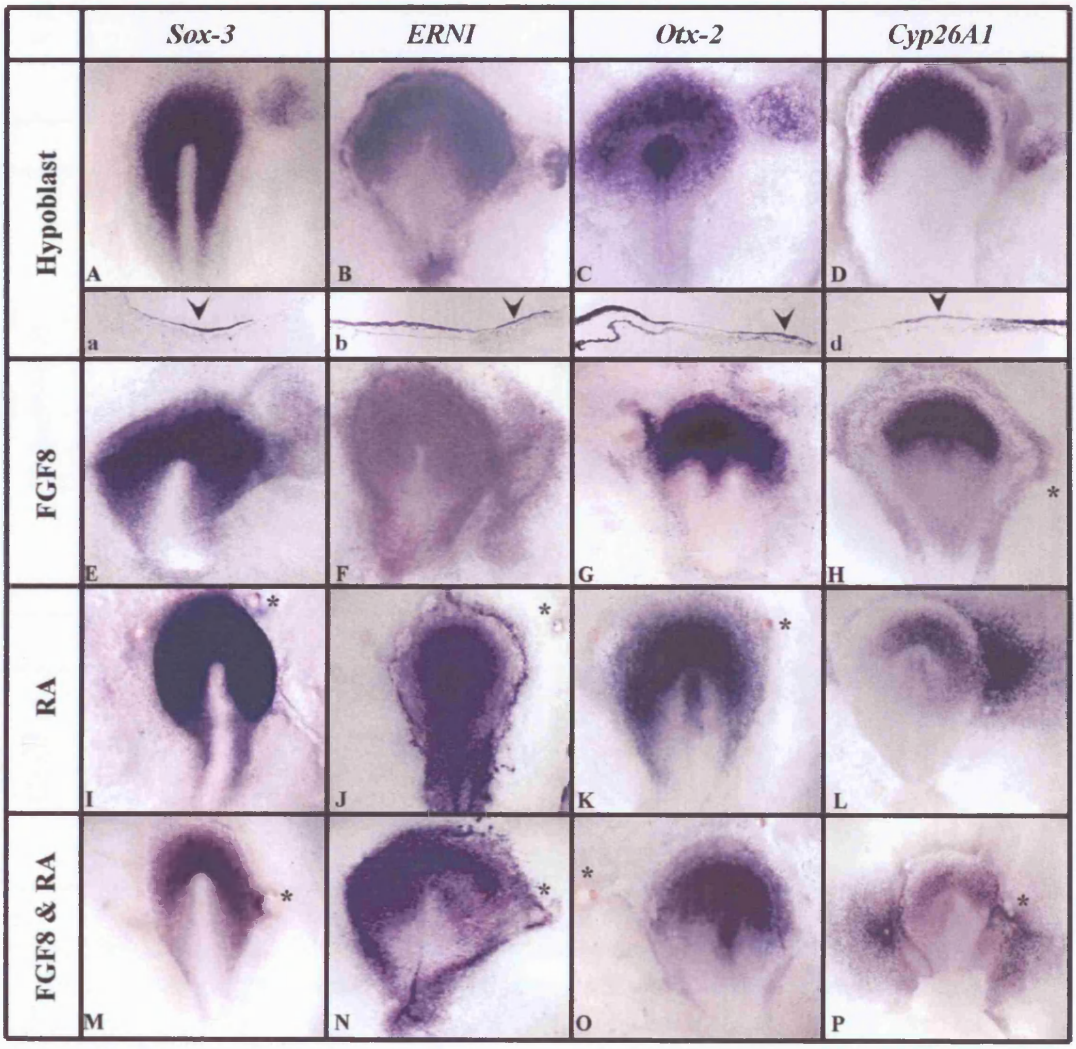


Figure 3.3 Hypoblast grafts incubated for 4-6 hours induce expression of *Sox3 (A), ERNI (B), Otx2* (C), and *Cyp26A1* (D). Sections through A-D reveal induced expression (arrow heads) in the ectoderm of the area opaca (a-d). FGF8 bead grafts induce *Sox3* (E), *ERNI* (F), and *Otx2* (G), but not *Cyp26A1* (H). RA bead grafts induce *Cyp26A1* (L), but not the other three markers (I-K). Grafting of adjacent FGF8 and RA beads reduces the expression of *ERNI* (N), and suppresses that of *Sox3* (M) and *Otx2* (O), as compared to single FGF8 beads. In P, a single RA bead (left) induces *Cyp26A1*, while an FGF8 bead rostral to an RA bead (right) blocks induction of *Cyp26A1* in its vicinity.

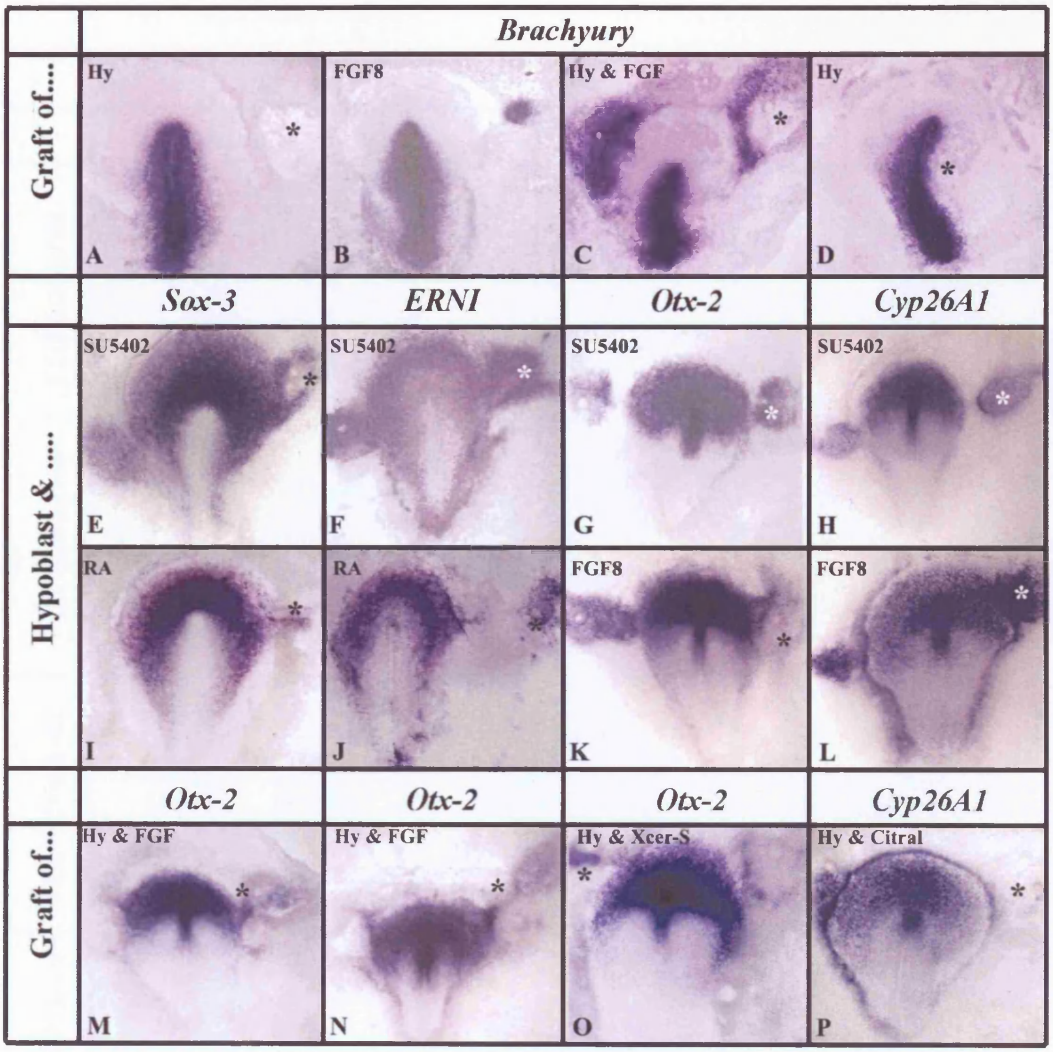


Figure 3.4 100µg/ml-FGF8 bead grafts induce *brachyury* (B, left side of C), while hypoblast (Hy) grafts do not (A), and can repress endogenous expression (D). Co-grafting of hypoblast blocks *brachyury* induction by an 100µg/ml-FGF8 bead (right side of C). *: sites of hypoblast grafts.

Co-grafting of SU5402 beads blocks hypoblast induction of *Sox3* (E), potentiates hypoblast induction of *Otx2* (G), and does not affect hypoblast induction of *ERNI* (F) or *Cyp26A1* (H). In E-H, hypoblast is grafted on both sides.

Co-grafting of a RA bead reduces but does not abolish hypoblast induction of *Sox3* (I) and *ERNI* (J). Co-grafting of an 50µg/ml-FGF8 bead reduces hypoblast induction of *Otx2* (K), and does not affect that of *Cyp26A1* (L). In K-L, hypoblast is grafted on both sides. Induced *Otx2* expression is continuous between adjacent grafts of hypoblast and 100µg/ml-FGF8 beads (junction marked by *), but does not form a complete ring (M-N; an FGF8 bead is also grafted without hypoblast on the left side).

Co-grafting of Xcer-S-expressing cells blocks 100µg/ml-FGF8 induction of *Otx2* (O, site of co-grafting marked by *; a single FGF8 bead is also grafted on the right).

Co-grafting of a citral bead blocks hypoblast indcution of *Cyp26A1* (P, site of co-grafting marked by *; a single citral bead is also grafted on the right).

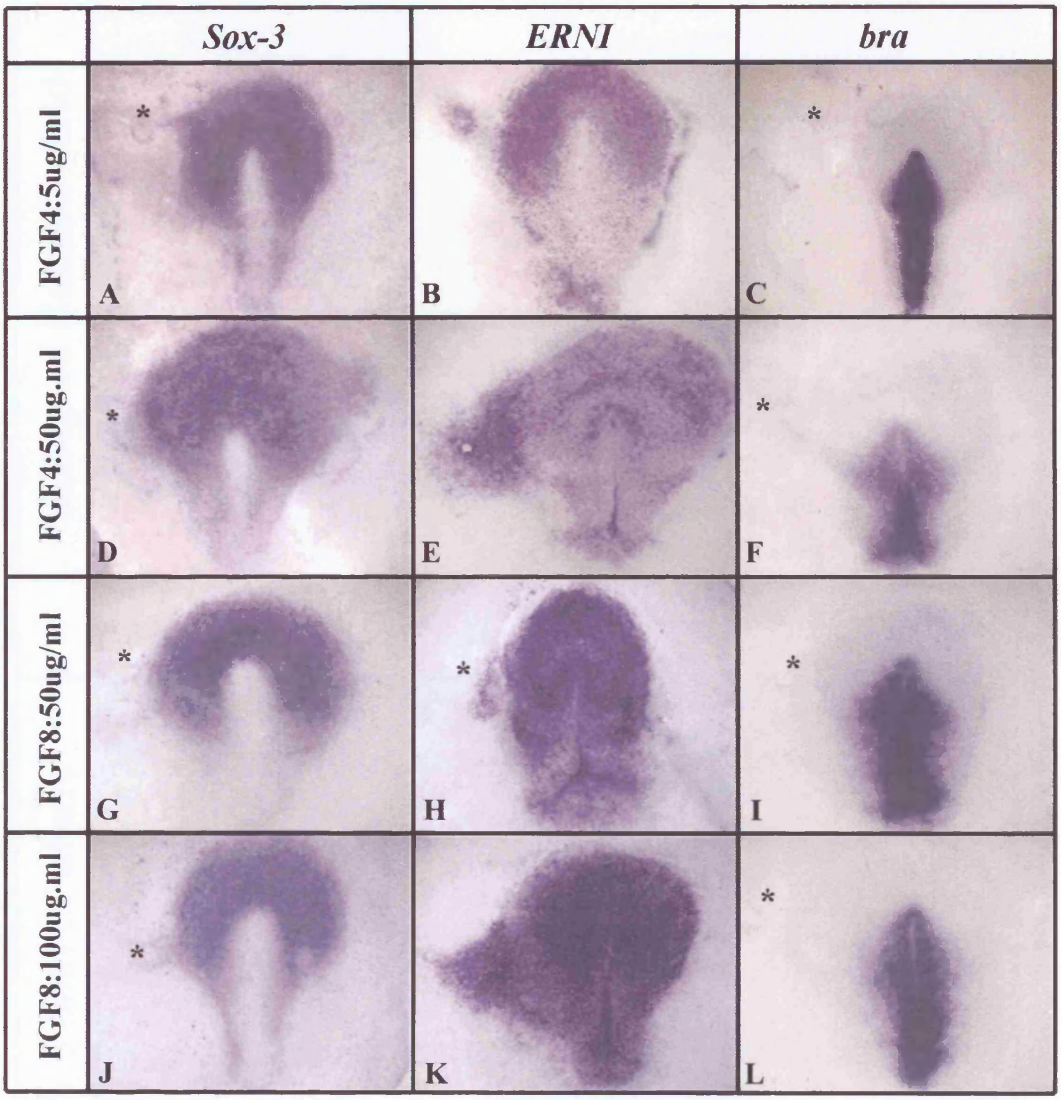


Figure 3.5 Identification of the concentrations of FGF4 and FGF8 at which *Sox3* and *ERNI* are induced without inducing *bra,* in 2 hours. 5µg/ml of FGF4 induces *ERNI* weakly (B) but not *Sox3* (A) or *bra* (C). 50µg/ml of FGF4 induces *Sox3* weakly (D) and *ERNI* strongly (E), without induction of *bra* (F). 50µg/ml FGF8 only induces *ERNI* and weakly (H), but at 100µg/ml FGF8 can induce *ERNI* strongly (K), *Sox3* at low levels (J), but still no *bra* is observed (L). (* indicates bead graft where little/no expression is induced).

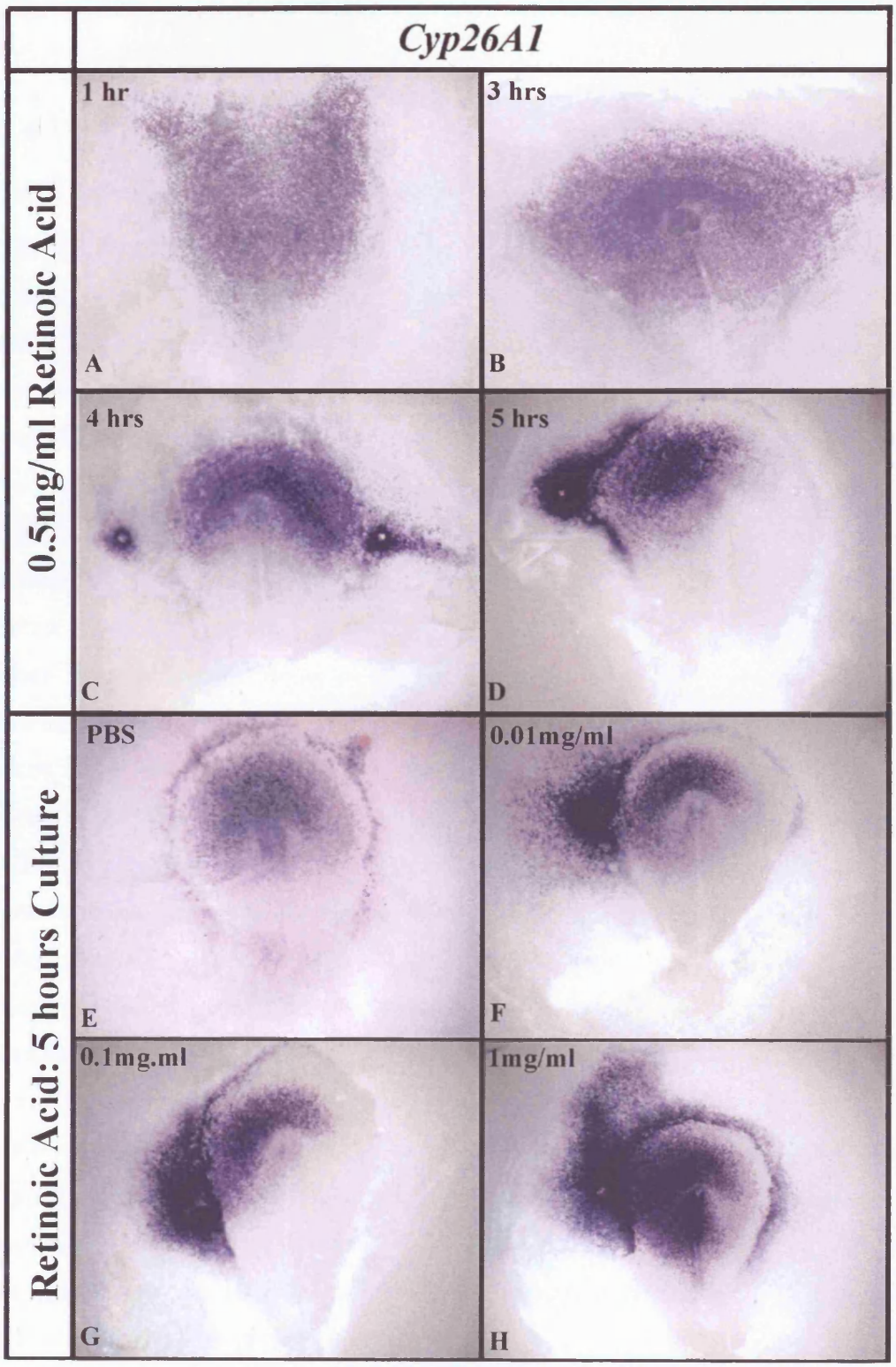


Figure 3.6 Time-course for the induction of *Cyp26A1* by 0.5mg/ml RA (A-D), revealing that it is induced within 1 hour and that the signal keeps increasing for 5 hours, and concentration-dependence of *Cyp26A1* induction by a 5h-incubation with a grafted RA bead (E-H), showing that the ectopic expression of *Cyp26A1* increases with the concentration of RA.

3.4 Discussion

3.4.1 The Hypoblast as a Transient Inducer

The hypoblast transiently induces *Sox3, ERNI* and *Otx2* when grafted in the area opaca (Foley et al., 2000; Streit et al., 2000). However, the dynamics of induction of these genes was unknown, including for how long the induction is maintained. A time course was performed to ascertain the induction and maintenance times for four markers induced by the hypoblast: *Sox3, ERNI, Otx2* and *Cyp26A1.* It was found that *Sox3* and *ERNI* are induced within 1-2 hours of a hypoblast graft whilst *Otx2* and *Cyp26A1* are expressed after 3-4 hours. This suggests that *Sox3* and *ERNI* might be induced by an early signal from the hypoblast and *Otx2* and *Cyp26A1* by a later, or different, signalling event. The expression of all genes is lost between 10 and 12 hours' incubation and does not reappear.

The state induced by the hypoblast was described as "pre-neural" (Foley et al., 2000; Stern, 2001) because *Sox3* later goes on to be expressed in the neural plate and *ERNI* is one of the first genes induced by a node graft (Streit et al., 2000) although these cells will not all become neural. The epiblast character could also be described as "pre-forebrain" (Foley et al., 2000; Stern, 2001) because the expression of *Otx2* later becomes restricted to the anterior neural plate and marks the prospective fore- and midbrain (Bally-Cuif et al., 1995), a restriction that develops concomitantly with the anterior movement of the hypoblast. *Cyp26*, which also becomes restricted to the rostral regions of the epiblast with the hypoblast, is required for anterior specification (de Roos et al., 1999; Abu-Abed et al., 2001; Sakai et al., 2001; Kudoh et al., 2002). Therefore, despite the fact that all the cells expressing these markers at the pre-streak stage will not go on to become anterior neural and the fact that the markers analysed are not exclusively 'anterior' or 'neural' indicators, a 'pre-neural' 'pre-forebrain' state would appear to be the best description.

The transient nature of the induction by the hypoblast suggests either that the signals emanating from the graft change over time and can no longer induce, or the grafted hypoblast has died after 10-12 hours and can no longer induce or maintain the initial induction, or that the induced area requires signals other than those supplied by the hypoblast to be maintained. The first possibility was tested by Foley et al. (2000)

who showed that a hypoblast taken from a stage 4 embryo is no longer able to induce *Sox3* or *Otx2* in the area opaca suggesting that the graft might lose the ability to induce those markers after several hours. However, here it has been shown that even when a young hypoblast is grafted into the area opaca and, following 8 hours' incubation, a second young hypoblast is grafted in the same place and incubated for a further 8 hours, the four markers studied are not maintained. Therefore the loss of induced expression in the host after 10-12 hours is unlikely to be due only to changes in the hypoblast. These results indicate that, in the embryo, signals from tissues other than the hypoblast might be required to maintain the expression of *Sox3, ERNI, Otx2* and *Cyp26A1*. This, and the time course over which the hypoblast does induce, correlate well with the movement of the hypoblast, a sheet covering the whole area pellucida to its restriction to the arc-shaped region located around the anterior edge of the embryo at stage 4: the germinal crescent (Vakaet, 1970; Stern and Ireland, 1981; Stern, 1990). At this time, the definitive endoderm is emerging from the streak and is partly responsible for displacing the hypoblast. In addition, the tip of the head mesendoderm including the prechordal mesendoderm (PME) appears in the midline adjacent to the future forebrain. The PME and head process are therefore candidate sources of signals that can maintain the initial expression of the markers analysed here and will be discussed in the next chapter.

3.4.2 The Molecular Nature of Hypoblast Signals

i. FGF4 and FGF8 can induce *Sox3, ERNI* and *Otx2* and FGF is required for the initial signalling by the hypoblast

The hypoblast can induce *Sox3* and *ERNI* within 1-2 hours. *FGF8* and *FGF3* have been detected in the pre-streak hypoblast (Streit et al., 2000) and the pre-streak area pellucida (Wilson et al., 2000) respectively. FGF8 is known to be able to induce *Sox3* and *ERNI* (Streit et al., 1998; 2000) and an early phase of FGF signalling has been suggested to be required for neural induction in the chick embryo (Wilson et al., 2000; Streit et al., 2000). Therefore the role of FGF signalling in the induction of these two genes by the hypoblast was investigated. FGF4 and FGF8 can induce both *Sox3* and *ERNI* in the area opaca within 2 hours without also inducing *brachyury*. Furthermore, when a hypoblast is grafted into the area opaca alongside a bead of FGF inhibitor, SU5402, the induction of *Sox3* is reduced (as judged by a reduction in

staining around the bead). *ERNI* is still expressed strongly, a result contrary to that observed when grafting SU5402 and a node graft which results in a lack of *ERNI* induction (Streit et al., 2000). This suggests that although FGF signalling is required for the hypoblast to induce *Sox3*, either the SU5402 used here is not able to fully block FGF signalling or another signal emitted by the hypoblast, which is not inhibited by SU5402, can also induce *ERNI*. This should be tested further however it is possible that another signal might present in the hypoblast that is not in the node, which is also capable of inducing *ERNI*.

Furthermore, it is not clear from these experiments whether FGF signalling is required within the hypoblast to activate a secondary signal to induce the epiblast or whether FGF signalling acts directly on the epiblast. This could be tested by electroporating a dominant negative FGFR construct into the area opaca followed by a hypoblast graft. This will reveal whether MAPK is activated in the responding tissue. A phosphorylated MAPK antibody could also be used to investigate whether the hypoblast activates the MAPK pathway in the area opaca.

ii. FGF induces *Otx2* in the area opaca but it is unlikely to be responsible for early epiblast expression

Whilst *Sox3* and *ERNI* are induced within 1-2 hours by both the hypoblast and FGF, *Otx2* is induced within 3-4 hours by the hypoblast and 5-6 hours by FGF4/8. Furthermore, unlike *Sox3* or *ERNI,* it requires a high concentration of FGF (50μg/ml FGF4 and 100μg/ml FGF8) to be induced. At these concentrations, *brachyury*, the mesoderm marker, is also induced. This suggests first that *Otx2* is not induced in the same early 'phase' of hypoblast signalling as *Sox3* and *ERNI*, and second that FGF cannot induce *Otx2* directly.

To test whether FGF is required for *Otx2* induction by the hypoblast, a hypoblast was grafted alongside a bead coated with SU5402. Instead of the decrease of induction observed in the same experiment for *Sox3*, an increase of *Otx2* expression was seen (as judged by stronger staining in the vicinity of the bead). This suggests that FGF needs to be down-regulated for a strong *Otx2* induction. In accordance with this, the over-expression of FGF, in combination with a hypoblast graft reduces the induction of *Otx2* compared to that induced by a hypoblast alone. This reduction is not the result of induction of *brachyury*, which is induced by FGF at high concentration, because the hypoblast represses such an induction.

If *Otx2* is down-regulated by FGF signalling then it is curious that FGF can induce *Otx2* expression in the area opaca. However, this might indicate that it is not FGF signalling in the hypoblast that induces *Otx2. Otx2* induced by the FGF bead might not be a marker of pre-neural epiblast. Indeed, *Otx2* is only induced by FGF at levels which also induce *brachyury* and when the latter's induction is inhibited with the Nodal-specific antagonist, XCerS (Piccolo, 1999), no *Otx2* expression is observed. Conversely, the hypoblast does not induce *brachyury* and yet does induce *Otx2*. In mouse, distinct regulatory elements control the expression of *Otx2* at different points in development and specifically, there is an epiblast enhancer and separate anterior neurectoderm enhancer (Kurokawa et al., 2004). Therefore, it is possible that the hypoblast activates the epiblast enhancer in an FGF-independent way whereas the anterior neurectoderm enhancer could be induced indirectly by FGF via the induction of *brachyury*. This could be tested by electroporating reporter constructs containing each of these enhancers in the area opaca to determine which one is induced by the hypoblast and which by FGF.

As well as being able to induce pre-neural, pre-forebrain markers, the hypoblast can prevent the induction of *brachyury* by FGF in the area opaca and the hypoblast can also repress the endogenous *brachyury* expression in the mesoderm emerging from the primitive streak. It is possible that this repression is a result of *Cerberus* expression in the hypoblast (Foley et al., 2000) that acts to inhibit Nodal signalling and therefore *brachyury* expression. Indeed, the hypoblast/AVE can antagonise primitive streak formation and this ability is mimicked in the chick by over-expressing *Cerberus* (Bertocchini and Stern, 2002; Perea-Gomez et al., 2002).

In *Xenopus* Carron et al. (2005) show that insulin-like growth factors (IGFs) can induce *Otx2* in the early gastrula embryo and that when either *IGF-1* or *Otx2* mRNA were injected into animal caps or ectoderm that was also injected with *activin* (to induce mesodermal markers), *Xbra* and *Xwnt11* expression was blocked but *Xwnt8* and *goosecoid* were not repressed. *In vivo* experiments revealed a similar result whereby *Otx2* and *IGF-1* can specifically repress *Xbra* and *Xwnt11* but not other mesodermal markers. *Xbra* induces *Xwnt11* that, in turn, drives the non-canonical Wnt pathway required for convergent extension movements (Tada and Smith, 2000; Heisenberg et al., 2000). The results from Carron et al. (2005) could therefore be compared to the induction of *Otx2* by FGF in the area opaca whereby *Otx2* is being

induced in a crescent shape by IGF in the anterior epiblast of the embryo proper in response to *bra* induction by the FGF bead.

The results here suggest that FGF signalling might have a role in the later expression of *Otx2* but it is unlikely to be involved in its induction by the hypoblast. In fact FGF inhibits the expression of *Otx2* induced by the hypoblast. *ERNI* expression is not prevented by inhibiting FGF signalling by the hypoblast suggesting that it too could also be induced by something else. Perhaps another factor, for example IGF, which has shown to be involved in head formation (Pera et al., 2001; Richard-Parpaillon et al., 2002; Eivers et al., 2004; Carron et al., 2005) could be acting in the hypoblast to induce both *Otx2* and *ERNI.*

iii Effects of different FGFs

In the experiments performed here, both FGF4 and FGF8 were used and the results for each were the same although not at the same concentrations. FGF4 was considered a more potent inducer of *brachyury* and would induce it at a concentration ten-fold lower than FGF8. *In vivo* the effects of FGF4 and FGF8 are not always the same and it tends to be context dependent. In the chick, the movements of the cells emerging from the primitive streak have been shown to be completely different in response to FGF4 compared to FGF8 (Yang et al., 2002). FGF8 in the primitive streak has been proposed as a chemorepellent for primitive streak mesoderm whilst FGF4 in the notochord was suggested to be a chemoattractant drawing paraxial mesoderm anteriorly and medially (Yang et al., 2002). In *Xenopus*, excess FGF8 can induce ectopic neurons but not axial or paraxial mesodermal markers and in fact it inhibits the expression of *Xbra* (Hardcastle et al., 2000). In the same study (and also Umbhauer et al, 2000) it was shown that FGF8 acts through FGFR4 to elicit posteriorisation directly, whilst eFGF (FGF4) seems to act through FGFR1. eFGF has been shown to posteriorise the neuraxis indirectly via induction of *Xcad3* and *Hoxa7* (Pownall et al., 1996). These results show that FGF4 and FGF8 can have diverse, context dependent roles. The hypoblast has not been shown to express *FGF4* although it does express *FGF8* (and perhaps other members of the family including FGF3 and its receptor, FGFR2, Wilson et al., 2000) (Streit et al., 2000). In the mouse, several FGFs are expressed before gastrulation including

FGF8 (Crossley and Martin, 1995), *FGF3/int-2* (Wilkinson et al., 1988), *FGF4* (Niswander and Martin, X), *FGF5* (Herbert et al., 2000; Haub and Goldfarb, 1991), *FGF17* and *FGF18* (Maruoka et al., 1998) and the receptor, *FGFR2* (Arman et al., 1998). Of these, *FGF3* and *FGF8* have been detected in the visceral endoderm (*FGF8* between 6.25 and 7.5 dpc) while *FGF4* is expressed in the inner cell mass and FGFR2 has been shown to be required for, but not expressed in, the visceral endoderm. Therefore, both the hypoblast and the AVE express *FGF8*. However, results from the mouse mutant for FGF8 reveal that it is not required for the formation of the AVE or for the induction of anterior neural markers *Six3* and *Hesx1* in the epiblast (Sun et al., 1999). Equally, mice null for *FGF3* do not have anterior defects and develop to term (Mansour, 1994). However, this might be due to an overlapping role of FGF3 and FGF8 or it could be that a different signalling molecule, like IGF, which shares a common pathway with FGF, might be required for AVE-mediated forebrain specification (Pera et al., 2002). Interestingly, Davidson et al (2000) have shown that 3-5 hours after application of exogenous FGF4 to the mid-late streak mouse embryo, expression of *brachyury* is expanded, *hesx1* is reduced both in the prospective anterior neurectoderm and the AVE but *Otx2* in the AVE and anterior neurectoderm remain unaffected. Only after 24 hours is the level of *Otx2* decreased. *FGF8* in the anterior neural ridge is the most sensitive of all rostral markers tested and is consistently absent following exposure to FGF4 (Davidson et al., 2000). The difference in the effect of FGF4 on *Otx2* here compared to the results we observe in the chick in which FGFs inhibit its induction by the hypoblast could suggest that other factors are involved in regulating *Otx2* or it could simply be indicative of the difference in experimental procedure. In the Davidson et al., (2000) study, whole embryos at mid-late streak are exposed to FGF4 whereas in our experiments, the ability of a hypoblast graft from a pre-streak embryo to induce *Otx2* is impaired when an FGF4 or FGF8 bead is applied locally.

In summary, it would appear that FGF can induce *Sox3* and *ERNI* directly within 1-2 hours and this is probably through an early phase of *FGF8* expression in the hypoblast. However FGF does not appear to be responsible for induction of *Otx2* or *ERNI* by the hypoblast. Indeed, it is possible that FGF in the hypoblast must be attenuated for *Otx2* to be induced. Exogneous FGF can repress *Otx2* induction by the hypoblast independently of induction of *brachyury*. At no point was *Cyp26A1*

expression observed in response to FGF but this is not surprising because FGF has been found to inhibit *Cyp26A1* in the zebrafish (Kudoh et al., 2002). This suggests that the early requirement for FGF signalling for neural induction (Wilson et al., 2000; Streit et al., 2000) could be mediated by the hypoblast. These results are particularly interesting because FGFs are known to be involved in caudalisation (Cox et al., 1995; Pownall et al., 1996; Muhr et al., 1997; 1999; Hardcastle et al., 2000; Koshida et al., 2002; Rentzsch et al., 2004 etc.).

iv. RA induces *Cyp26A1* but a combination of RA and FGF result in a down-regulation in expression of all the genes analysed.

RA, as well as FGF, is generally considered to be a caudalising factor (Blumberg et al., 1997; Chen et al., 2001; Dupe and Lumsden, 2001; Kaiser et al., 2003; Shiotsugu et al., 2004; Molotkova et al., 2005) however, it has been implicated in the early steps of epiblast specification (Knezevic and Mackem, 2001). The epiblast requires the apposition of the hypoblast at pre-streak stages for expression of *Not1* and *Not2* and these two genes can be induced ectopically by RA (Knezevic and Mackem 1995; 2001). This indirectly suggests that the hypoblast produces RA. Although *RALHD2* has not been observed in prestreak embryos at stage XII by RT-PCR (Wilson et al., 2000) it was detected at stage 4 in the hypoblast by mRNA *in situ* hybridisation (Halilagic et al., 2003). The hypoblast induces *Cyp26A1* and, since this has also been shown to be induced in response to RA (Martinez-Ceballos et al., 2001; Dobbs-McAuliffe et al., 2004), RA is a candidate. Indeed, RA does induce *Cyp26A1* very strongly in the area opaca and furthermore, the RALHD2 antagonist, citral (Kikonyogo et al., 1999: Berggren et al., 2001) can prevent the ability of the hypoblast from inducing *Cyp26A1* indicating that RA is normally produced by the hypoblast and that, in the embryo, *Cyp26A1* is up-regulated in the epiblast where it might protect it from the caudalising influences of RA, since *Cyp26A1* is a catabolising enzyme for RA (Sonneveld et al., 1999). This is consistent with current data. It has been shown in zebrafish that *Cyp26* over-expression anteriorises the axis by the removal of the caudalising influences of RA (Kudoh et al., 2002) and in mouse that it acts along the A-P axis to establish an uneven distribution in the levels of RA and hence the varying the amount of caudalisation (Sakai et al., 2001). However, in *Xenopus* it has been shown that injection of a morpholino targeted against αRAR at the two cell stage results in both anterior and posterior truncations

with the most severe phenotypes occurring when the MO is distributed dorsally (Shiotsugu et al., 2004) suggesting that RA signalling is required for initial dorsalisation and then must be repressed. Therefore there might be an early requirement for RA (Shiotsugu et al., 2004), as for Wnt signalling (Sokol et al., 1995; Baker et al., 1999; Liu et al., 1999; Wessely 2001; Kuroda et al., 2004) and FGF signalling (Wilson et al., 2000; 2001; Streit et al., 2000) for neural induction and head development.

FGF and RA can induce all the factors under study: FGFs induce *Sox3, ERNI,* and *Otx2*; RA induces *Cyp26A1*. A combination of RA and FGF applied in the area opaca might therefore be expected to induce the whole range of markers. This was found not to be the case and, in fact, the expression levels of all markers were reduced. *Sox3, ERNI* and *Otx2* inductions were all decreased to just a few cells expressing these markers following grafting of FGF and RA beads in the area opaca and when an RA bead was grafted with a hypoblast, the hypoblast-mediated induction of *Sox3* also resulted in less intense staining. This suggests that either an excess of RA inhibits the signalling ability of FGF or in combination RA and FGF induce a different set of genes. Both RA and FGF have been implicated in caudalisation (RA: Blumberg et al., 1997; Chen et al., 2001; Dupe and Lumsden, 2001; Kaiser et al., 2003; Shiotsugu et al., 2004; Molotkova et al., 2005, FGF: Cox et al., 1995; Pownall et al., 1996; Muhr et al., 1997; 1999; Hardcastle et al., 2000; Koshida et al., 2002; Rentzsch et al., 2004) and it is possible that caudal neural markers might be induced (FGFs have been shown to induce early posterior neural tissue: Storey et al., 1998).
Alternatively, because RA can induce node and notochord marker, *cNot1,* ectopically (Knezevic and Mackem, 1995; 2001) and the hypoblast can also induce *cNot1* when grafted in the area opaca (personal observations) it is possible that a mis-expression of FGF and RA results in mesodermal rather that pre-neural marker induction. However, the induction of *cNot1* does not necessarily indicate a pre-mesendodermal fate. After all, it is also expressed later in the developing forebrain (Stein and Kessel, 1995).
The gene induced by RA, *Cyp26A1*, is reduced upon the addition of FGF. Its expression in the area opaca, induced by the RA bead, comes to an abrupt halt at the point at which it meet the adjacent FGF-bead. This is interesting because it indicates two possibilities for FGF and RA signalling. Firstly, FGF might block RA signalling

and therefore no induction of *Cyp26A1* would be observed. This seems unlikely because RA, in combination with FGF, can affect the targets of FGF signalling. The second possibility is that FGF is repressing *Cyp26A1*. Since *Cyp26A1* degrades RA, this would provide a permissive environment to allow RA to activate its other targets. This idea is supported by the findings in zebrafish showing that *Cyp26* can be blocked by over-expression of either Wnt or FGF which results in a posteriorisation of the embryo whilst over-expression of *Cyp26* causes an anteriorisation (Kudoh et al., 2002). Therefore, it seems likely that FGF might provide a permissive environment for RA-mediated caudalisation by blocking *Cyp26A1* up-regulation.

FGF and RA have been shown to interact in many situations both agonistically and antagonistically. In the developing limb bud, RA can down-regulate *FGF4* expression and yet leave *FGF8* unaffected (Hayes and Morriss-Kay, 2001) and in the pre-somitic mesoderm FGF signalling can repress *RALDH2* in the somitic mesoderm and, conversely, RA can repress *FGF8* in the pre-somitic mesoderm (Diez del Corral et al., 2003). Cross regulation of RA and FGF has also been documented in *Xenopus* (Shiotsugu et al., 2004). Shiotsugu et al. (2004) report that blocking the FGF pathway using XFD, either in whole embryos or in animal caps, results in a down-regulation of *XRARα* and *RALDH2* expression and that conversely, injection of a morpholino against RAR produces embryos in which *FGFR4* is expanded laterally in the anterior regions but it, and *FGFR1* are strongly down-regulated posteriorly. Taken together, these data suggest that FGF and RA signalling pathways do interact but context and timing are critical to the outcome.

To speculate, it would seem that the hypoblast expresses *FGF* and within the first 1-2 hours of a graft and this induces *Sox3* and *ERNI*. *Otx2* is then induced after 3-4 hours by an unknown signal from the hypoblast. *Cyp26A1* is also induced at this point suggesting that RA is either not active in the hypoblast from the beginning of the grafting, or that initial FGF signalling represses *Cyp26A1*. In the latter case, RA might have a critical signalling role very early on, in the first 1-2 hours of hypoblast-mediated inductions.

v. Wnt antagonists do not induce *Sox3. ERNI, Otx2* or *Cyp26A1* in the area opaca and do not appear to enhance the inducing abilities of FGF or RA.

Wnt signalling has many roles in early embryonic patterning. In the 'two inhibitor' model, both Wnt and BMP must be antagonised in order for a forebrain to be induced (Glinka et al., 1997). Wnt has also been strongly implicated as a posteriorising factor of the neuraxis: in the chick, increasing levels of WNT3A result in an increasingly caudalised anterior neural plate suggesting that Wnt acts in a concentration-dependent fashion (Nordstrom et al., 2002). Interestingly, WNT3A cannot posteriorise stage 4 rostral forebrain explants without the cooperation of FGF8. The concentration of FGF8 did not affect the regional character of the neural genes induced but it seemed to act permissively to enable Wnt3a to caudalise (Nordstrom et al., 2002). Similarly, in *Xenopus*, XWNT8 and XWNT3A have concentration-dependent abilities to caudalise the neurectoderm (Kiecker and Niehrs, 2001).

However, Wnt signalling is also thought to be required for the initiation of neural induction. Injection of *dishevelled* (required for Wnt signal transduction) mRNA into prospective ventral mesoderm cells results in a dorsalisation of the axis whilst injection into ectodermal cells induces anterior neural differentiation (Sokol et al., 1995). It was also shown that Wnt8 can inhibit *BMP4* at gastrulation stages in *Xenopus* (Baker et al., 1999) and that β-catenin has a role in activating BMP antagonists, such as Chordin, in the dorsal side of the embryo (Wessely et al., 2001) suggesting that the promotion of dorsal structures and neural induction could be effected through BMP inhibition and the activation of BMP antagonists by Wnt signalling.

Apparently contradictory evidence comes from the pre-gastrula chick embryo in which FGF-mediated neural induction of medial epiblast cells is blocked in the lateral epiblast by Wnt signalling, allowing BMP to convert the cells to an epidermal fate (Wilson et al., 2001). However, the timing of the Wnt signal is most likely critical and in the mouse mutant for *Wnt3*, no neural induction or regional specification is observed despite AVE markers being expressed (Liu et al., 1999) suggesting that Wnt is required as an early step to dorsalise the embryo prior to neural induction.

Wnt3 is expressed in the posterior visceral endoderm in the mouse (Liu et al., 1999). In the chick, *Wnt1, Wnt3a, Wnt5a, Wnt8c* and *Wnt11* have been observed in the avian

pre-streak embryo and both *Wnt5a* and *Wnt8c* are strongly expressed in the area opaca (Skromne and Stern, 2001; Wilson et al., 2001; Chapman et al., 2004) although the only one reported to be expressed weakly in the hypoblast at XII is *Wnt11* (Skromne and Stern et al., 2001). *Wnt3a* and *Wnt8c* have been detected in lateral regions of stage X-XIII embryos by RT-PCR and not in medial regions (Wilson et al., 2001) but it is unclear whether this is in the epiblast or lower layer. Wnt antagonists are expressed in the lower layer at pre-streak stages including *Dkk1, crescent* and the multi-functional inhibitor *Cerberus* (Pfeffer 1997; Zhu et al., 1999; Foley et al., 2000; Skromne & Stern 2001, Chapman et al., 2002).

It is possible that the induction of any genes by the hypoblast requires inhibition of Wnt signalling in the area opaca. Therefore, it was investigated whether Wnt antagonism alone can induce *Sox3, ERNI, Otx2* or *Cyp26A1* in the area opaca but this was not observed. Following the reports from Wilson et al. (2001), it might be expected that Wnts must be blocked for FGF signalling to be able to result in neural induction. Therefore, Wnt antagonists and FGF beads were grafted in the area opaca. However, the induction of *Sox3* and *ERNI* remained the same as with FGF grafted alone. When Wnt antagonists and RA were grafted into the area opaca, *Otx2* was not induced.

It would be interesting to see whether *Otx2* is still expressed as a crescent shape when FGF and Wnt antagonists are combined. It would also be worthwhile to test a combination of RA, FGF and Wnt antagonists, adding the various factors at different time-points to try to recreate the conditions of the hypoblast. The hypoblast expresses Wnt antagonists (Foley et al., 2000; Chapman et al., 2002) and so it should be checked as to whether a hypoblast grafted into the area opaca could still induce the markers studied if Wnt is over-expressed. *Wnt8c* and *Wnt5a* (Skromne and Stern, 2001) are already expressed in the area opaca but they do not prevent the induction by the hypoblast or by FGF. The levels of Wnt signalling or their effectors might not be the same in the area opaca compared to the posterior side of the embryo proper and so it could be more informative to mis-express one of the down-stream targets of Wnt signalling in the area opaca in order to see an effect on the ability of the grafts to induce.

To summarise, Wnt antagonists have not been shown here to have a role in hypoblast-mediated inductions. However, these results are not comprehensive and further experiments are required to investigate their role in early development.

3.4.3 The Hypoblast's place in models of A-P patterning

The hypoblast can induce a pre-neural, pre-forebrain state in the area opaca (this study and Foley et al., 2000; Streit et al., 2000). This transient activation of the epiblast is the first step in the revised 'activation-transformation' model (Stern, 2001). However, it does not fit so well with the original version by Nieuwkoop and Nigtevecht (1954) in which the activation step induces anterior neural character. In their model, the 'activation' gradient was determined to be strongest just caudal to the level of the PME and head process because grafts of ectoderm placed here produced the greatest quantity of neural tissue. This is much later than the time at which the hypoblast/AVE has an effect.

However, the hypoblast can be accommodated into the general idea of some of the other models to a certain extent. Hypoblast rotation experiments show that it acts to orchestrate the movements of the overlying prospective forebrain to follow in the same direction as it is spreading (Waddington, 1930; 1932; 1933; Foley et al., 2000) and has been proposed to be required to move the prospective forebrain away from caudalising signals from the node (Foley et al., 2000; Kimura et al., 2001). Beads coated in RA and placed in a posterior-lateral position can also result in the tip of the streak and the prospective head territories being reoriented but in this case they develop away from the RA (Knezevic and Mackem, 2001) indicating that this movement is mediated by the hypoblast in response to a strong source signals emanating from the posterior of the embryo.

In the mouse *Cripto* mutant the mesodermal structures do not form and the AVE, although molecularly normal, is not displaced anteriorly and remains at the distal tip (Liguori et al., 2003). The anterior neural plate develops and expresses all the correct markers but it also remains at the distal tip of the embryo (Liguori et al., 2003). Likewise, in the zebrafish *Cripto* mutant, OEP (one-eyed pinhead), the prospective forebrain does not end up in the rostral position (Gritsman et al., 1999; Feldman et al., 2000). This suggests that the forebrain is normally displaced anteriorly in response to signals from the posteriorly developing primitive streak and organizer and that the hypoblast/AVE might be the recipient of these signals that then act to direct the prospective head region away from them. Both the AVE and hypoblast have the ability to repress primitive streak formation mediated by the expression of Nodal antagonists (Bertocchini and Stern, 2002; Perea-Gomez et al., 2002). In mouse

Nodal induces proliferation in the distal visceral endoderm, which is suppressed in the AVE once *Cerberus* and *Lefty1* are expressed (Yamamoto et al., 2004). This sets up a differential of proliferation that results in the more proximal-posterior VE displacing the AVE towards the future anterior pole. Therefore, Nodal might act indirectly to displace the AVE/hypoblast and the source of Nodal antagonists away from the site of primitive streak formation. As it is displaced, the prospective forebrain is directed concomitantly (Waddington, 1930; 1932; 1933; Foley et al., 2000).

The ability of the hypoblast/AVE to direct the movement of the prospective forebrain could be compatible with the ideas of Yamada's (1950) 'double potency' model which states that the combination of morphogenetic movements and molecular signals are important in patterning the embryo. This is consistent with some of the functions of the hypoblast. The hypoblast directs the movement of the prospective forebrain away from the forming primitive streak and, presumably, in its wake, other cells will come closer to those signals and develop more posterior character. However, this does not take into account the active process of induction by the hypoblast of the epiblast through vertical signalling. The hypoblast would appear to prepare the epiblast for further signals from the primitive streak and node because the hypoblast can recapitulate the first steps of induction of pre-neural genes of a node graft (Streit et al., 1998; 2000; Knoetgen et al., 2000; Sheng et al., 2003).

Vertical signalling from the hypoblast to the epiblast is reminiscent of the qualitative model whereby distinct regions of underlying mesoderm have 'organizer' ability and impart specific A-P neural identity to the ectoderm. The hypoblast is by no means an organizer since it can induce neither definitive neural nor an anterior neural state in the area opaca (this study; Knoetgen et al., 1999; Foley et al., 2000; Streit et al., 2000). Equally the AVE is not a true head organizer because it fails to induce anterior neural character when grafted alone into an ectopic position (Tam and Steiner, 1999) and although it can induce rostral neural marker *Otx2* it is not able to maintain neurectodermal expression (Ang et al., 1994; Acampora et al., 1998; Rhinn et al., 1998). However, the hypoblast/AVE does have an active role in specifying the character of the epiblast (Thomas and Beddington, 1996; Acampora et al., 1998; Rhinn et al., 1998; Knoetgen et al., 1999; Shawlot et al., 1999; Foley et al., 2000; Streit et al., 2000) mediated by an early phase of FGF signalling and potentially RA and Wnt signalling, which have all been suggested to be required initially for neural

induction and/or anterior specification (FGF: Streit et al., 2000; Wilson et al., 2001, RA: Knezevic and Mackem, 2001; Shiotsugu et al., 2004, Wnt: Sokol et al., 1995; Baker et al., 1999; Liu et al., 1999; Wessely 2001).

In summary, the transient activation of a pre-neural, pre-forebrain state in the epiblast by the hypoblast, and the requirement of the AVE for head formation does not fit comfortably with any of the original models for A-P patterning but the revised model for 'activation-transformation' (Stern, 2001) accommodates this by suggesting that hypoblast/AVE, 'activates' the epiblast in a step prior to, but required for, neural induction and anterior specification.

Chapter 4: Signals maintaining the expression of genes induced by the hypoblast

4.1 Introduction

The previous chapter has shown that, consistent with the Stern (2001) model, the hypoblast can transiently induce the expression of several pre-neural, pre-forebrain genes in naïve ectoderm. The results suggest that both FGF and RA can mimic and are required for some of this function but a role for Wnt antagonism was not uncovered. The induction of marker gene expression disappears after about 10-12 hours and even a second hypoblast graft cannot maintain it. This is consistent with the results obtained by Foley (2000) who showed that the avian hypoblast is unable to maintain an induction after overnight culture. The rabbit hypoblast is also able to induce the anterior neural marker, *Ganf,* when grafted into the area pellucida/area opaca border of a host chick embryo (Knoetgen et al., 1999) although it is unclear whether this induction is transient because the embryos were always analysed within 6-10 hours of incubation due to the deterioration of the grafted tissue. Therefore, it is possible that the mammalian hypoblast, like its chick counterpart, is only capable of transient induction. The transient nature of the induction of the markers analysed could suggest that in the chick embryo, once the hypoblast is displaced anteriorly to the germinal crescent, the anterior ectoderm requires and receives stabilising, neuralising signals from other tissues. Hence, after this time (stage 4), the continued presence of the hypoblast is no longer sufficient to maintain the expression of pre-neural genes (Foley et al., 2000). Following gastrulation, head mesendoderm emerges from the node as a mixed population of PME and head process cells that separate into distinct regions by stage 5, with the head process fully extended by stage 6-7. The PME, head process and anterior definitive endoderm (ADE) have all been proposed to play a role in head development (as discussed in Chapter 1). Once the PME has emerged from the organizer, the latter is no longer able to induce a secondary axis that includes rostral markers suggesting that the PME precursors must be resident in the organizer to confer this ability (in chick: Storey et al., 1992; Dias and Schoenwolf, 1990; Foley et al., 1997, in mouse: Beddington, 1994; Kinder et al., 2001, in zebrafish: Saúde et al., 2000). Indeed, in the chick, this ability can be

restored by recombining the node with the PME. Also, although the PME is unable to induce forebrain structures when grafted alone in the area opaca (Foley et al., 1997) it is able to change the regional specification of more posterior neurectoderm (prospective hindbrain) to produce a vesicle of forebrain character (Foley et al., 1997; Pera and Kessel, 1997), effectively a "rostralisation". In mouse, if the PME is removed from explants of neural plate, the forebrain marker *Nkx2.1* is lost (Shimamura and Rubenstein, 1997). The PME is also critical in amphibian head development. In the salamander, Mangold (1933) showed that the region between the PME and anterior chordamesoderm has the greatest ability to induce ectopic anterior neural structures, whilst in *Xenopus,* ablation of the PME results in anterior defects (Schneider and Mercola, 1999). These embryological data provide evidence that the PME is involved in anterior neural specification.

The PME secretes both Wnt antagonists (DKK1: Glinka et al., 1998; Chapman et al., 2004, CRESCENT: Pera and de Robertis, 2000) and BMP antagonists (Noggin: Smith and Harland, 1992; Jones et al., 1992, Bachiller et al., 2000), suggested by Glinka et al. (1997) to be required for head formation in the 'two inhibitor' model (discussed in Chapter 1). The PME also expresses *Chordin* when it first emerges from the node but, in the chick, this is subsequently down-regulated in the PME by BMP signalling from the anterior definitive endoderm (Vesque et al., 2000). However, it is maintained in the head process (Vesque et al., 2000), which could partly explain why head process but not PME can induce neural tissue in the area opaca (Foley et al., 1997; Rowan et al., 1999).

BMP inhibition is required to promote neural fate (Hemmati-Brivanlou and Melton, 1997; Pera et al., 2001; 2003; Linker & Stern 2004; Delaune et al., 2004; Reversade et al., 2005). Both in chick and in *Xenopus*, over-expression of BMP2 or BMP4 even after gastrulation suppresses neural markers suggesting that its inhibition may be required after the initial stages of neural induction to maintain neural character (Hartley et al., 2001; Linker et al., 2004). Mice lacking both *chordin* and *noggin* have almost a complete absence of forebrain, suggesting that inhibition of BMP (by more than one antagonist) is required for anterior neural development (Bachiller et al., 2000) although the stage(s) at which BMP must be antagonised are unknown. However, the formation of anterior neurectoderm might not be as simple as

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inhibition of BMP because *BMP7* in chick (Dale et al., 1997) and *BMP4* and *BMP7* in *Xenopus* (Hartley et al., 2001) are expressed in the PME. BMP7 and SHH secreted by the PME have been shown to ventralise the forebrain in chick (Dale et al., 1997) and the double *chordin/noggin* mouse mutant has reduced SHH expression in the PME which might lead to some of the observed anterior neural defects (Anderson et al., 2002). It is likely that the balance between BMPs and BMP antagonists produced by the PME and head process act in concert both to maintain an anterior neural character and selectively to ventralise specific regions of the developing CNS (Hartley et al., 2001).

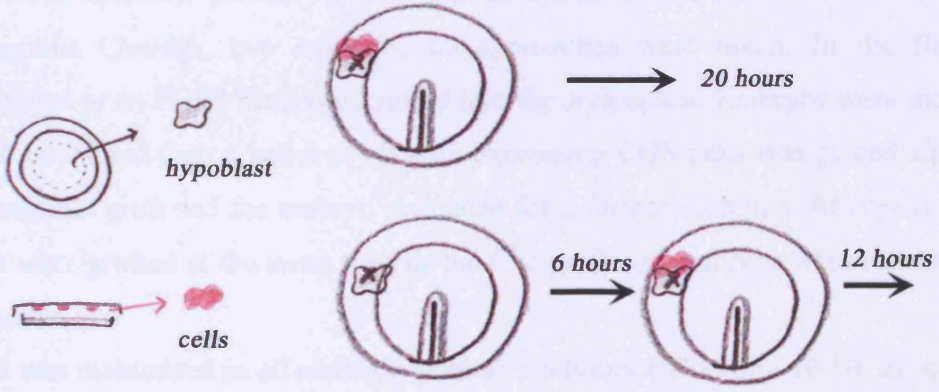
In the 'two-inhibitor' model (Glinka et al., 1997) Wnt antagonism was suggested to be required for head development as well as BMP antagonism. There might be an early requirement for Wnt signalling to dorsalise the ectoderm before neural induction (Sokol et al., 1995; Baker et al., 1999; Liu et al., 1999; Bainter et al., 2001), although in chick explants Wnt prevents neuralisation in favour of epidermal fate and this has been suggested to act by preventing FGF from acting as a BMP antagonist (Wilson et al., 2001; Wilson and Edlund, 2001). Wnts have also been shown to act in a concentration-dependent context to caudalise the anterior neurectoderm (Moon et al., 1997; McGrew et al., 1997; Kiecker and Niehrs, 2001; Nordstrom et al., 2002) and their inhibition is required for head development (Glinka et al., 1998; Mukhopadhyay et al., 2001, Houart et al., 2002; Lagutin et al., 2003; Nambiar et al., 2004). Mukhopadhyay et al. (2001) demonstrated that, in chimaeric mutants for *Dkk1,* this Wnt antagonist is required in the anterior axial mesendoderm for anterior neural specification and not in the AVE. Furthermore, injection of *Dkk1* in *Xenopus* embryos can expand the prechordal region and the forebrain and co-injection with dominant-negative BMP receptor (tBR) produces ectopic prechordal plates and second heads (Glinka et al., 1998) revealing roles of Dkk1 both for anterior mesendoderm development and in the neural plate to specify anterior character (Kazanskaya et al., 2000). An addition to the 'two-inhibitor' model was made when Piccolo et al. (1999) reported that Nodal antagonism is also required for head development but this is most likely to be indirect, through its role in concentration-dependent mesoderm induction (Osada et al., 1999; Gritsman et al., 2000; Feldman et al., 2000; Thisse et al., 2000; Agathon et al., 2003; Yamamoto et al., 2004).

Thus, the tissues most likely to have a role in maintaining the induced pre-neural, pre-forebrain state created by the hypoblast are the PME, ADE and head process, all of which express BMP and Wnt and Nodal antagonists including *chordin* in the node and chordamesoderm (Streit et al., 1998; Dale et al., 1999; Vesque et al., 2000; Anderson et al., 2002), *Dkk1* in the node, chorda- and prechordal mesoderm (Mukhodophyay et al., 2001; Chapman et al., 2004 and personal observations), *crescent* in the anterior definitive endoderm and prechordal endoderm (Pfeffer et al., 1997; Chapman et al., 2002; 2004) and *cerberus* in the anterior definitive endoderm (Zhu et al., 1999; Chapman et al., 2002; 2004). Therefore, these genes are candidates as maintenance factors and are tested in this Chapter for their ability to maintain hypoblast-mediated inductions in the area opaca. Indeed Chordin can maintain a transient induction of *Sox3* induced by a short-term node graft (Streit et al., 1998; discussed further in Chapter 1) or by FGF8 (Streit et al., 2000). However, none of the factors have been analysed for their ability to maintain the expression of genes induced by signals from the hypoblast. In this chapter, hypoblasts are grafted with cells secreting the factors mentioned above, either alone or in combination, and the maintenance of the four markers shown to be induced by the hypoblast (in the previous chapter), *Sox3, ERNI, Otx2* and *Cyp26A1*, checked for sustained expression. The results show that, in combination with a hypoblast graft, BMP and/or Wnt antagonism are able to maintain *Sox3* and *ERNI,* and induce a neural plate-like morphology in the area opaca, whilst both factors are required to maintain *Otx2* but this is still not sufficient to induce definitive neural marker, *Sox2*. Nodal antagonism appears to diminish the maintenance of the three markers. Finally, *Cyp26A1*is maintained by RA. Therefore, it would seem that BMP antagonism *or* Wnt antagonism is required to maintain a pre-neural state and the combination of the two is necessary for the maintenance of a pre-forebrain state; however, further maintenance factors responsible for specifying a definitive neural state remain to be identified.

4.2 Materials & Methods

4.2.1 Grafting

In the following experiments, either an FGF bead (25µg/ml FGF8, which did not induce *brachyury*) or a hypoblast was grafted into the area opaca of a host as described in the previous chapter. The culture was either incubated for 6 hours and then a pellet of COS cells grafted alongside the initial graft and returned to the incubator for a further 12 hours or the pellet of cells and the hypoblast/FGF8 bead were placed at the same time and the embryo cultured for 18-20 hours (see below).



The cell pellets generated were produced to secrete between 1 and 4 different proteins. The initial experiments (4.3.1i) were performed using pellets of 1500 transfected cells. In subsequent experiments, the number of cells transfected with each factor was maintained at 500 whilst the pellet was formed of 1500 cells, made up with 1000 mock-transfected cells for *chordin*, *XCerbS* or *Cerberus* alone, 500 mock- 500 *Dkk1*- and 500 *cres*-transfected cells for Wnt-antagonist experiments and 500 of each, *chordin*-, *Dkk1*- and *crescent*-transfected cells for Wnt- and BMP-antagonism. However, when all four factors were used, although 500 cells of each were incorporated into the pellet, the proportion of each one was less (1/4 rather that 1/3 as before).

It is important to note that the cell pellets often stain during the mRNA detection procedure and it can appear as if there is an induction or maintenance of the gene of interest. It is observable under the microscope when this is the case but embryos were sectioned to confirm.

4.3 Results

4.3.1 Maintenance of Hypoblast-mediated Induction by Wnt, BMP and Nodal antagonists

i. Chordin can maintain *Sox3* expression transiently induced by either the hypoblast or FGF but only generates a neural plate morphology in combination with the hypoblast

Both FGF and the hypoblast can induce *Sox3* expression transiently in the area opaca (previous chapter). To test whether this induction can be maintained by the BMP antagonist Chordin, two experimental approaches were taken. In the first, the hypoblast or an FGF8 bead was grafted into the area opaca. Embryos were incubated for 6 hours and then a pellet of *chordin*-expressing COS cells was placed alongside the original graft and the embryo incubated for a further 12 hours. Alternatively, the cells were grafted at the same time as the first graft and embryos were incubated for 18 hours.

Sox3 was maintained in all cases (hypoblast + 6 hours + Chordin: 10/10; hypoblast + Chordin: 10/10; FGF8+6 hours +Chordin: 8/8; FGF8+Chordin: 8/8) (Fig.4.1). The intensity of the staining and the size of the region stained with *Sox3* following a hypoblast graft was much stronger (20/20)(Fig.4.1A,B) than following a FGF8 bead graft (16/16) (Fig.4.1C,D). (Chordin-expressing cells alone were not able to induce Sox3: 0/5). Histological sections revealed that induction of *Sox3* by the hypoblast was not only maintained with the addition of Chordin but the morphology of the ectoderm changed, becoming thickened and the cells columnar, resembling a neural plate (Fig.4.1 a,a',b,b'). In certain places above the cell pellet graft the expression of *Sox3* was reduced, mimicking the expression in the neural plate over the notochord, where *chordin* is expressed (Fig.4.1a,b). This thickened morphology was not observed when FGF beads were used instead of the hypoblast (Fig.4.1c,d). Therefore, Chordin can maintain the induction of *Sox3* by either a hypoblast or FGF8. However, what appeared to be a neural plate-like morphology was obtained only in embryos that had received a hypoblast graft, suggesting that the hypoblast produces additional signals required to instruct a morphological change. Furthermore, the induced region of *Sox3* covers a larger area of area opaca than that directly apposed to the cell pellet. This occurs in 100% of the embryos although the

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extent varies. 60% (12/20) of cases exhibit a slightly wider (less than 150%, as measured on the section) expression domain compared to the cell pellet (Fig.4.1 a) whilst the other 40% have a much wider domain (150-200%) than the pellet (Fig. 4.1b). This suggests that signals might spread in the plane of the ectoderm.
There was no observable difference between waiting for 6 hours between grafts and grafting both items at the same time (Fig.4.1, compare A with B and C with D) and so for subsequent experiments, all grafts were performed at the same time.

ii. Chordin maintains *ERNI* but not *Cyp26A1* or *Otx2* expression induced by the hypoblast

Sox3 induction was maintained when Chordin-secreting COS cells were added to a hypoblast graft (Fig.4.2A). *ERNI* was also maintained (6/6) (Fig.4.2 B) but expression of both *Cyp26A1* (0/6) and *Otx2* (0/6) was lost as if the hypoblast had been grafted alone (Fig.4.2 C,D). Chordin-expressing cells alone were not able to induce any of the markers (0/5 for each marker). This indicates that BMP antagonism is able to maintain, but not induce, pre-neural markers *Sox3* and *ERNI* but it is not sufficient to maintain the pre-forebrain markers *Otx2* and *Cyp26A1*.

iii. Wnt antagonists can maintain hypoblast-induced *Sox3* and *ERNI* but not *Otx2* or *Cyp26A1*

Sox3 and *ERNI* were maintained by the BMP-antagonist Chordin but the other two markers analysed were not maintained. To test whether Wnt antagonism might maintain these inductions, the same experiment was repeated using cell pellets secreting both DKK1 and CRESCENT. Once again, *Sox3* (5/5) and *ERNI* (5/6) are maintained (Fig.4.2 E,F) but *Cyp26A1* (0/6) and *Otx2* (0/8) are not (Fig.4.2 G,H). Wnt-antagonist-expressing cells were not able to induce any of these markers when grafted without a hypoblast (0/5 for all markers). Therefore, neither Wnt- nor BMP-antagonism can maintain the latter two markers but both Chordin and Wnt antagonists are able to maintain *Sox3* and *ERNI*.

iv. A combination of BMP- and Wnt-antagonists can maintain hypoblast-induced expression of *ERNI, Sox3* and *Otx2* but not *Cyp26A1*

Neither Wnt- nor BMP-inhibition, along with the hypoblast, could maintain the expression of *Otx2* or *Cyp26A1* and therefore a combination of the two inhibitions was tested. The same experiment was repeated as above but using COS cell pellets secreting Chordin, DKK1 and CRESCENT. *Sox3* (5/5) and *ERNI* (5/5) were still maintained (Fig.4.2 I,J). *Cyp26A1* expression was once again lost (8/8) (Fig.4.2 K). However, this time, *Otx2* expression was also maintained (5/8)(Fig.4.2 L). Since *Otx2* is transiently expressed by the organizer, embryos were tested for induction of *brachyury* expression but this was absent (0/8). Once again, cell pellets secreting inhibitors of both pathways grafted without a hypoblast were not able to induce any of the markers tested (0/6 for all markers). Therefore, a combination of the two antagonists is able to maintain the expression of *Otx2* induced by the hypoblast.

v. Nodal antagonism has a weak ability to maintain the hypoblast-induced expression of *Sox3* and *ERNI* only

Although BMP- and Wnt-antagonism in combination was able to maintain *Otx2* expression, *Cyp26A1*, induced by the hypoblast, was still lost. Therefore, a different signalling pathway, Nodal, was inhibited by adding pellets of XCerS-secreting COS cells to the hypoblast grafts. Following overnight incubation the embryos did maintain expression of *Sox3* (3/6) and *ERNI* (2/6) although the intensity of staining for these two genes was not as strong as for any of the three combinations tried above and the proportion of embryos in which expression was maintained is lower (Chordin: *Sox3*: 100%; *ERNI*: 100%, DKK1/CRES: *Sox3*: 100%; *ERNI*: 83%, Chordin+DKK1+CRES: *Sox3*: 100%; *ERNI*: 100%, XCerS: *Sox3*: 50%; *ERNI*: 33%). Neither Cyp26A1 (0/5) nor Otx2 (0/5) were maintained (Fig4.2M-P). Cells expressing the Nodal-antagonist grafted without a hypoblast did not elicit induction of any of the markers (0/4 *Sox3*, *ERNI*; 0/5 *Otx2*, *Cyp26A1*). Therefore, although Nodal-antagonism can maintain the expression of *Sox3* and *ERNI*, its ability to do so is much weaker than for BMP- and/or Wnt-antagonists.

vi. Nodal inhibition abolishes the maintenance of *Otx2* expression by BMP- and Wnt-antagonists

Finally, a combination of all the secreted factors was tried and COS cell pellets were prepared to secrete Chordin, DKK1, CRESCENT and XCer-S. These pellets were grafted together with a hypoblast, as above. Once again, *Sox3* (6/6) and *ERNI* (4/6) were maintained. The intensity of staining of these two markers was stronger than with XCerS and hypoblast alone but not as strong as for the grafts of the hypoblast plus Wnt- and/or BMP-antagonists (Fig.4.2 Q,R). *Cyp26A1* (0/8) and *Otx2* (0/8) were not maintained (Fig.4.2 S,T). This could indicate that Nodal antagonism blocks the maintenance of *Otx2* by Wnt- and BMP-inhibition. However, although the absolute number of cells secreting each factor has remained constant in this experiment compared to above, the proportion of each with respect to the total number of cells is reduced from 1/3 to ¼ and therefore there might be some dilution. However, when the full length *Cerberus* expression construct, which should inhibit Nodal, Wnt and BMP signalling pathways (Piccolo et al., 1999) was transfected into COS cells which were then grafted with a hypoblast, none of the markers were maintained (0/6 for each marker). This suggests that *Cerberus* might prevent BMP- and Wnt- inhibition from maintaining the expression of induced markers.

vii. The expression of *Cyp26A1* is maintained by RA and does not require a hypoblast graft

In all of the above experiments, it has never been possible to maintain the expression of *Cyp26A1* induced by the hypoblast in an overnight culture. In the previous chapter, RA could induce the expression of *Cyp26A1* and therefore, RA-coated beads were grafted in the area opaca and embryos were incubated for at least 18 hours. RA was able to maintain this expression, even without a prior hypoblast graft although the number of embryos with expression was greatly reduced with the lowest concentration of RA tested (1 μg/ml) despite the fact that this concentration can induce *Cyp26A1* in 100% of embryos after 5 hours' incubation (see Chapter 2) (0.1mg/ml: 4/4; 0.01mg/ml: 4/5; 0.001mg/ml: 1/5). Therefore, continued, relatively high levels of RA are sufficient to maintain the hypoblast-induced expression of *Cyp26A1*.

4.3.2 Maintenance of early markers is not sufficient to induce *Sox2*

In the experiments above using a combination of Wnt-, BMP- and Nodal-antagonists it was possible to maintain *Sox3*, *ERNI* and even, in the case of simultaneous Wnt- and BMP-inhibition, *Otx2*, as well as to induce an early neural plate-like morphology. Does this neural plate-like thickening express the earliest definitive neural marker, *Sox2*? As above, a hypoblast was grafted with cells expressing either Chordin (Fig.4.3 A), DKK1 and CRESCENT (Fig.4.3 B), Chordin, DKK1 and CRESCENT (Fig.4.3 C), XCerS (Fig.4.3 D) or Chordin, DKK1, CRESCENT and XCerS (Fig.4.3 E). The embryos were incubated for 18 hours and then processed for *in situ* hybridisation. In no case was any *Sox2* expression observed (0/20, 0/8, 0/8, 0/8 and 0/8, respectively). Therefore, despite maintaining pre-neural and pre-forebrain markers (except *Cyp26A1*) and producing a neural plate-like morphology, a definitive neural state was not induced by any combination of these factors together with a hypoblast, as assessed by expression of *Sox2*.

Figure 4.1 Chordin can maintain the expression of *Sox3* that is transiently induced by a hypoblast or FGF8

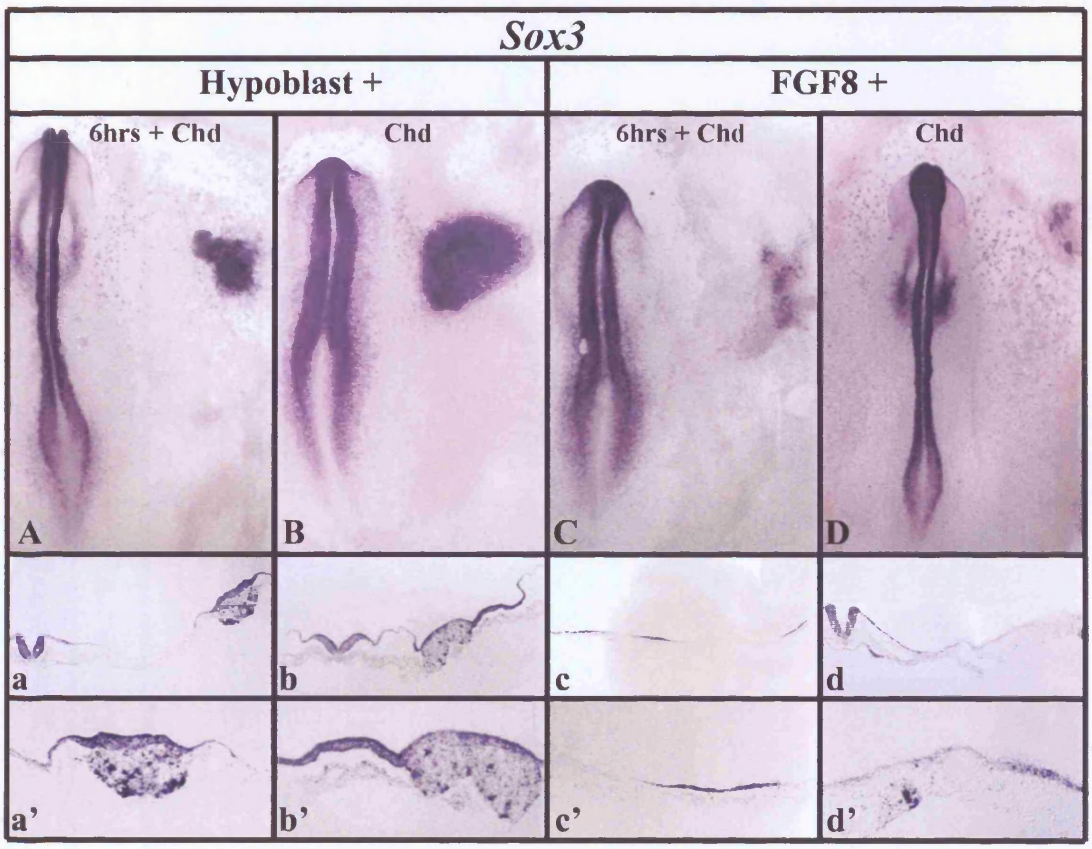


Figure 4.1 Chordin can maintain the expression of *Sox3* induced by a hypoblast or FGF8 bead. In A and B hypoblast grafts are used whilst in C and D FGF8 beads are used to transiently induce *Sox3*. A and C have been grafted with hypoblast/FGF8 and incubated for 6 hours before adding Chordin-expressing cells. In B and D, all grafts were performed at the same time. In all cases, *Sox3* is maintained. Sections were cut (a-d) and the region of the graft is enlarged in a'-d'. When hypoblasts were grafted (A,B) the ectoderm overlying the graft thickened and showed a neural plate-like morphology (a',b') but this was not observed when FGF8 beads were used (C,D and c',d').

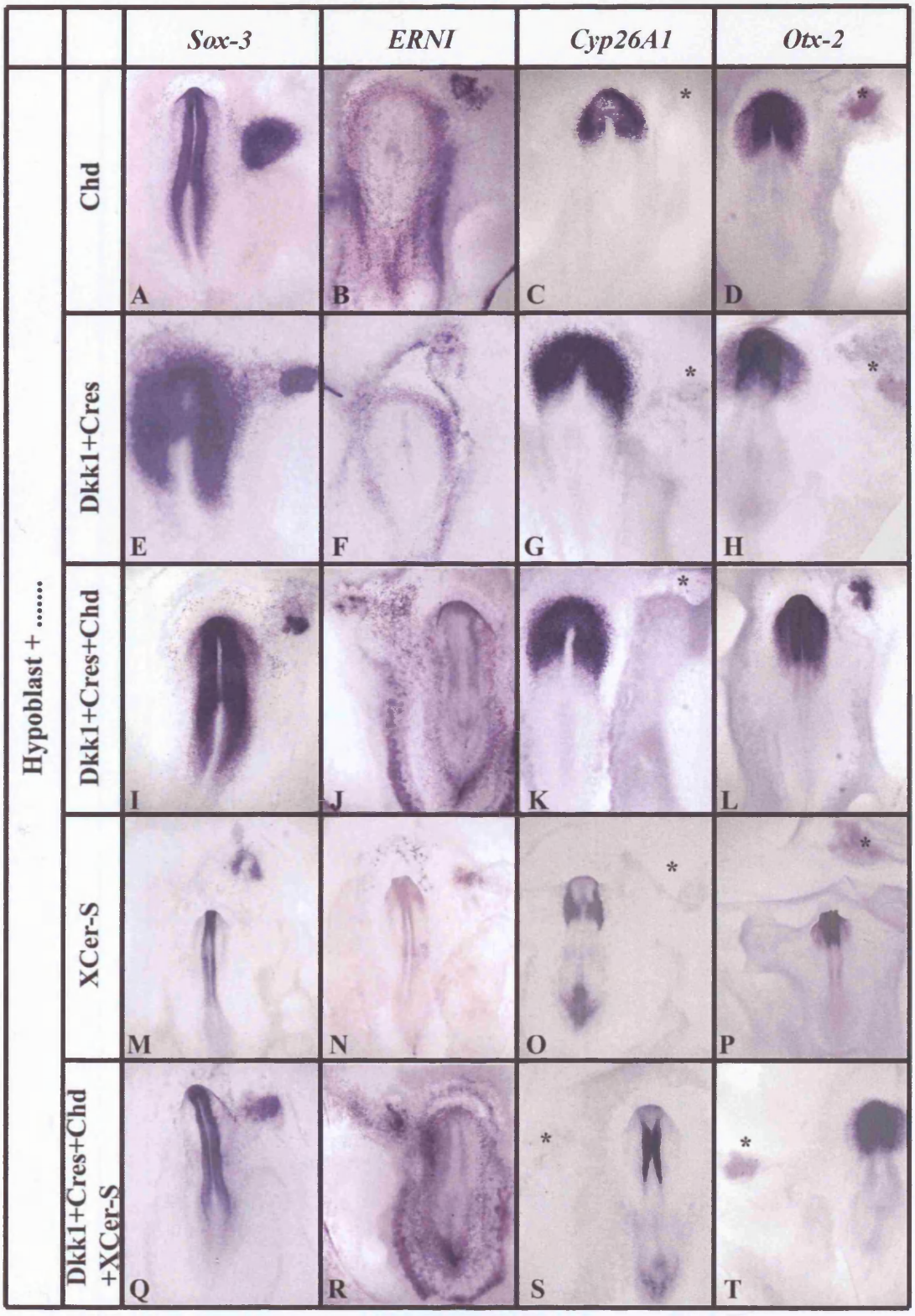


Figure 4.2 The maintenance of genes induced by the hypoblast. *Sox3 (A)* and *ERNI (B)* are maintained by *Chordin*-expressing cells, *Dkk1+Crescent* (E,F), a combination of the two (I,J), *Xcer-S* (M,N) and all factors together (Q,R). *Cyp26A1* is never maintained (G,K,O,S) and *Otx2* is only maintained by a combination of Chordin and Dkk1+crescent (L). * indicates position of graft when no induction is observed.

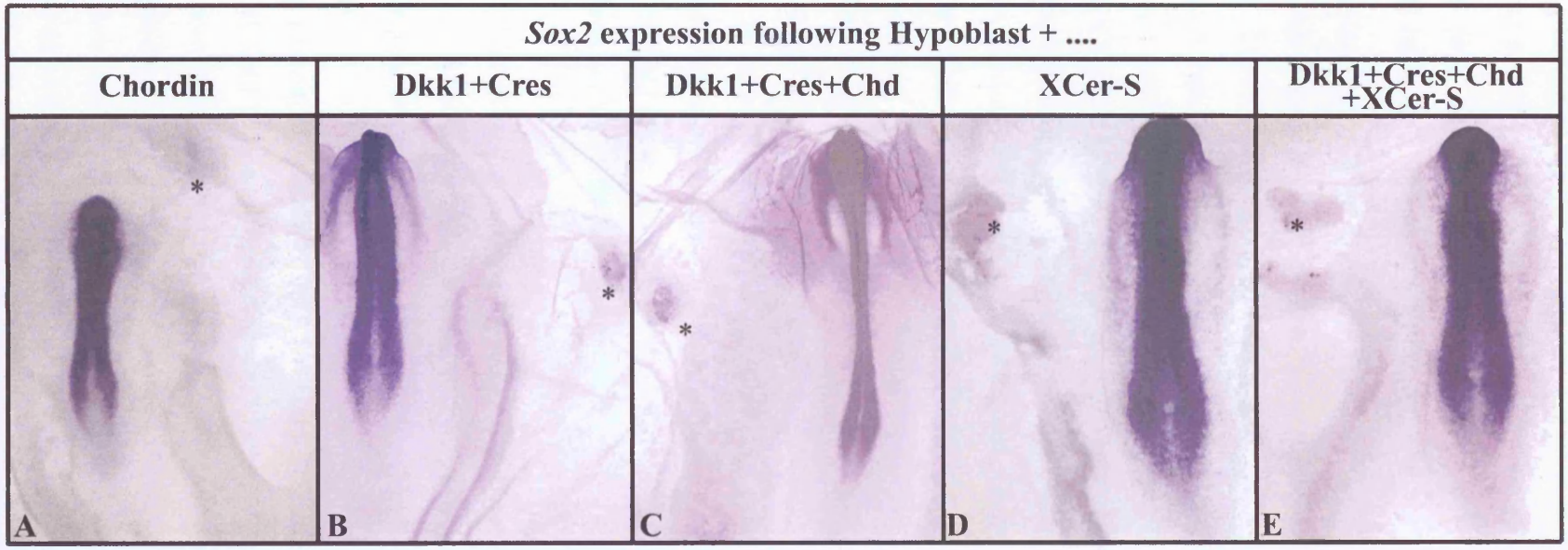


Figure 4.3 *Sox2* cannot be induced by a combination of hypoblast graft and either *Chordin* (A), *Dkk1+Crescent* (B), a combination of these (C), *XCer-S* (D) or all factors together (E). * indicates position of graft

4.4 Discussion

4.4.1 Transiently induced expression of pre-neural, pre-forebrain markers by the hypoblast can be maintained by additional secreted factors

The hypoblast can induce *Sox3, ERNI, Otx2* and *Cyp26A1* in the area opaca. However, this induction is transient and disappears after 10-12 hours (Chapter 3 and Foley et al., 2000; Streit et al., 2000). It has been shown that a transient expression of *Sox3* induced following a brief exposure (3-5 hours) to a grafted Hensen's node (Streit et al., 1998) or an FGF8-bead (Streit 2000) can be maintained by BMP-antagonist, Chordin. In this Chapter, the molecular nature of the signals required to maintain the transient induction by the hypoblast were investigated using the BMP-antagonist Chordin, Wnt antagonists Dkk1 and Crescent and the Nodal antagonist XCerS. The results reveal that either BMP- or Wnt-antagonism can maintain only hypoblast-induced *Sox3* and *ERNI*; Nodal inhibition can occasionally and weakly maintain their expression. A hypoblast graft and the combination of inhibiting both the BMP and Wnt signalling pathways results in the continued expression of *Otx2*. *Cyp26A1* can be maintained by RA even without prior induction by the hypoblast. All transiently induced factors can be maintained but in no case was induction of the definitive neural marker *Sox2* observed, suggesting that maintenance of the early markers is not sufficient for neural induction.

BMP-inhibition in combination with a hypoblast results in the responding tissue becoming thickened and the cells columnar, resembling an early neural plate. Furthermore, the ectoderm directly apposing the cell pellet secreting Chordin is often *Sox3* negative. This can be compared to the neural plate overlying the *chordin*-expressing notochord, which is also *Sox3* negative (Rex et al., 1997). A transient induction of *Sox3* by a node grafted for 3-5 hours and then removed, followed by a graft of *Chordin*-expressing cells, can be maintained and this too results in a neural plate morphology in the ectoderm (Streit et al., 1998). This neural plate morphology does not cover such a large region of the area opaca as that following a hypoblast plus a *Chordin*-expressing cell graft. This could be due to the fact that the grafted hypoblast spreads in the area opaca and therefore transiently induces a larger region

than a node graft. The thickness of the neural plate-like morphology is greater when *Chordin*-expressing cells maintain the transient induction from a hypoblast than from a node; however, hypoblast grafts are left for 10-12 hours while the node graft only remains in contact with the host for 3-5 hours, which leaves open the question of whether it is the tissue (hypoblast as compared to node) or the time in contact that is responsible for this difference.

Chordin-expressing cells are also able to maintain *Sox3* transiently induced by FGF8 (this Chapter and Streit et al., 2000). However, when FGF8 is used as the transient activator prior to BMP antagonism there is no morphological change in the ectoderm. The hypoblast alone does not induce a thickening in the area opaca (Fig. 3.3 a-d) but it does so when combined with BMP antagonism, suggesting that its induction of markers in the area opaca is not reducible to FGF signalling and that other signals from the hypoblast are required to elicit a morphological neural plate.

4.3.2 The PME, ADE and head process are candidate tissues for producing the signals required to maintain hypoblast inductions

The tissues that underlie the anterior neurectoderm once the hypoblast has been displaced are the PME, ADE and head process. Furthermore, these tissues have all been implicated in head development. Precursors of the PME are required in the node for it to induce an axis that expresses rostral neural markers in all systems studied to date (Spemannn, 1931; 1938; Dias and Schoenwolf, 1990; Storey et al, 1992; Foley et al., 1997; Schneider and Mercola, 1999; Saúde et al., 2000; Kinder et al., 2001). The PME is required for anterior neural development. In *Xenopus*, inhibition of Dkk1 using antibodies against the protein results in a diminished PME and associated forebrain defects, whereas overexpression of *Dkk1* and *tBR* can induce an ectopic PME and concomitantly, an ectopic head (Glinka et al., 1998; Kazanskaya et al., 2000). In the mouse, Nodal$^{-/\Delta 600}$ mutants were generated to lack a 600bp region containing two binding sites for FoxH1; the resulting embryos fail to form correct anterior definitive endoderm and prechordal mesoderm. The lack of these tissues results in impaired anterior neural development (Robertson et al., 2003). In the chick, the PME can induce an ectopic forebrain vesicle in the embryonic ectoderm (Pera and Kessel, 1997; Foley et al., 1997) although it is not able to induce anterior neural tissue in the area opaca (Foley et al., 1997). The PME emerges from the node with the head process and these two tissues resolve into distinct regions of

head mesoderm, expressing different markers (Dale et al., 1997; 1999; Vesque et al., 2000). The ADE has been proposed to be instructive in eliciting the differences in gene expression in the PME compared to head process (Vesque et al., 2000). The head process in chick and the anterior notochord in *Xenopus* are able to induce anterior neural markers (Hemmati-Brivanlou et al., 1990; Rowan et al., 1999) and the removal of the entire anterior midline (including the PME and head process) leads to a loss of head structures in mouse (Camus et al., 2000) suggesting that they are required to maintain these structures. The anterior axial mesendoderm has also been shown to be required for the maintenance of *Otx2* expression in the neurectoderm following its initial activation by the AVE (Ang et al., 1994; Acampora et al., 1995; Rhinn et al., 1998). A requirement for the ADE in anterior neural development has also been shown in the chick by Withington et al. (2001) who performed a series of ADE ablation experiments to uncover a role for it in the maintenance of forebrain markers. Therefore, the head mesendoderm and the ADE are required for the maintenance of the anterior neurectoderm.

Wnt- and BMP- antagonism are able to maintain hypoblast-induced *Sox3, ERNI* and *Otx2* with the latter marker requiring a combination of both. The PME and head process both initially express BMP-antagonists, *chordin* and *noggin* (Connolly et al., 1997; Dale et al., 1999; McMahon et al., 1998; Hongo et al., 1999; Bachiller et al., 2000; Anderson et al., 2002) as well as the Wnt antagonists *Dkk1* and *crescent* (Pfeffer et al., 1997; Kazanskaya et al., 2000; Pera and de Robertis, 2000; Mukhopadhyay et al., 2001; del Barco Barrantes et al., 2003). However, once the PME and head process separate, the PME loses expression of *chordin* and starts expressing *BMP7*, essential for ventral forebrain patterning in co-operation with SHH (Dale et al., 1997; 1999) although it does maintain expression of the BMP-antagonist, *noggin* (Smith and Harland, 1992). The ADE also expresses the Wnt antagonist, *crescent* (Pfeffer et al., 1997) as well as Wnt-, BMP- and Nodal antagonist, *Cerberus* (Piccolo et al., 1999; Zhu et al, 1999) and transiently expresses *BMP2, BMP4, BMP7* (Vesque et al., 2000). Therefore, the young PME and head process and the ADE could be the source of maintenance signals (BMP- and Wnt-antagonists) and *noggin* persists even after the PME loses *chordin* expression, suggesting that even though BMP7 in the PME ventralises the forebrain (Dale et al., 1999; Vesque et al., 2000), BMP inhibition emanating from the PME may have other functions.

Gain and loss of function experiments suggest that the secretion of BMP and Wnt antagonists is necessary for correct head development. In *Xenopus,* Dkk1 can induce an ectopic PME in combination with BMP antagonism and also an ectopic head (Glinka et al., 1998) and the mouse *dkk1* mutant (Mukhopadhyay et al., 2001) as well as double mutants for *chordin/noggin* (Bachiller et al., 2000; Anderson et al., 2002) and *dkk1/noggin* (del Barco Barrantes et al., 2003) have anterior neural defects. However, the prior requirement of these antagonists in the formation of the mesendoderm cannot be ruled out as indirectly responsible for the failure in head formation. In the mouse *Six3* mutant, the prosencephalon is truncated and there is a concomitant expansion of *Wnt1* expression (Lagutin et al., 2003). SIX3 can negatively regulate WNT1 directly and its overexpression in the zebrafish *headless/tcf3* mutant rescues the rostral neural defects (Lagutin et al., 2003). However, some antagonism of Wnt signalling also propagates within the plane of the ectoderm; for example *Tlc,* which is expressed in the anterior boundary of the neural plate in zebrafish, acts in a concentration-dependant manner to inhibit Wnt signalling and promote telencephalic fate (Houart et al., 2002).

In the experiments performed here using a combination of Wnt-, BMP- and Nodal-antagonists in collaboration with a hypoblast graft, *Sox3* and *ERNI* were maintained in almost all instances and *Otx2* was maintained by BMP- and WNT-inhibition but in no case was *Cyp26A1* maintained. RA was, however, able both to induce and to maintain *Cyp26A1* expression after overnight culture with no prior requirement for a hypoblast graft. This might seem inconsistent with the transient nature of the induction of *Cyp26A1* by the hypoblast alone. When beads soaked in the lowest concentration of RA were grafted in the area opaca, the induction of *Cyp26A1* was either maintained weakly or not at all after overnight culture, suggesting that the exogenous RA had been exhausted or degraded (perhaps by the CYP26A1) and there is no feedback of RA to maintain the expression of *Cyp26A1*. The PME and ADE express *RALDH2* (Halilagic et al., 2003) and would therefore provide a continued source of RA in anterior regions of the embryo, and the RA would presumably become degraded in the regions of *Cyp26A1* expression.

Hence, there is circumstantial evidence for RA and its inhibititors, and for Wnt- and BMP- antagonists in the PME, head process and ADE being involved in the maintenance of a pre-neural, pre-forebrain character in the epiblast and it would be worth trying spatially and temporally restricted gain and loss of function experiments

in these tissues for Wnt, BMP and RA signalling to elucidate their role in this maintenance.

4.3.3 Maintenance of a pre-neural, pre-forebrain state does not result in neural induction

The 'default model' proposes that BMP inhibition alone is sufficient for neural induction (Hemmati-Brivanlou and Melton, 1997, discussed in the introduction). Recently, it was shown that morpholino-mediated knockdown of three BMP antagonists, Chordin, noggin and follistatin results in the loss of all dorsal structures in *X. tropicalis* (Khokha et al., 2005) illustrating the importance of BMP inhibition for dorsoventral patterning. A role for FGF signalling to inhibit BMP via the phosphorylation of Smad1 has been proposed (Pera et al., 2003). The activation of MAPK and its phosphorylation has been proposed to be responsible for neural differentiation of dissociated *Xenopus* ectoderm (Kuroda et al., 2005) originally thought to be the result of a dilution of BMP signals (Munoz-Sanjuan and Hemmati-Brivanlou, 2002). However, inhibition of BMP signalling is not the only role of FGF in neural induction: BMP/SMAD1 inhibition can suppress epidermal fate in the non-neural ectoderm but it cannot result in neural induction without low-level eFGF signalling (Linker and Stern, 2004; Delaune et al., 2005). A similar, although not entirely consistent result was found in the chick epiblast explants (Wilson et al., 2000: 2001), where FGF can inhibit *BMP4* expression (required for medial pre-streak epiblast explants to acquire expression of neural markers). However, Wnt, normally only expressed in the lateral epiblast, which differentiates into epidermis in culture, was reported to prevent the repression of BMP4 by FGF. However, neither inhibition of BMP nor over-expression of FGF could elicit a neural fate in non-neural ectoderm (Wilson et al., 2000; 2001). Furthermore, Linker and Stern (2004) showed that a whole range of BMP antagonists used in combination (Chordin, Noggin, Smad6 and dominant negative BMP receptor) are unable to induce *de novo* expression of *Sox3* and they are unable to induce *Sox2* when grafted with FGF-coated beads (FGF2, FGF3, FGF4 or FGF8) in the area opaca *in vivo*.

The results presented in this chapter show that whilst both BMP- and/or Wnt antagonism can maintain *Sox3* and *ERNI*, and *Otx2* can be maintained by their combined inhibition, prior transient induction (by the hypoblast, FGF or a node graft:

Streit et al., 2000) is required as a prerequisite for this expression and at no time is *Sox2* induced. Even though FGF8 and Chordin combined can maintain the expression of *Sox3*, a neural plate morphology is not observed (unlike when using Chordin with the hypoblast or node (Streit et al., 1998; 2000)) suggesting that other factors are required to induce an early neural plate and also indicating that even a combination of FGF and BMP- and /or Wnt antagonist is not sufficient for neural induction.

Glinka et al. (1997) proposed a "two-inhibitor" model for head induction that requires the simultaneous antagonism of Wnt and BMP signalling. Although the embryonic region chosen to perform the experimental procedure in *Xenopus* and in chick experiments is not equivalent: injection of Wnt- and BMP-antagonists were injected into the 4-cell stage *Xenopus* embryo whereas experiments in this thesis are performed in the area opaca at a much later equivalent stage. However, the present data do not fit this model. Wnt- and BMP- inhibition, even together, do not induce any of the four markers tested here. In combination with the hypoblast they can maintain *Sox3, ERNI* and *Otx2* but this does not lead to neural induction. The modification of this model to include Nodal antagonism (Piccolo et al., 1999) was tested but the combination of Nodal-, Wnt- and BMP-inhibitors either with or without the hypoblast was still unable to induce *Sox2*. Likewise, FGFs, even in combination with BMP-, Wnt- and Nodal-antagonists, were insufficient to induce *Sox2* expression in the chick study by Linker and Stern (2004). The addition of the Nodal inhibitor, XCerS (Piccolo et al., 1999) to a hypoblast graft, as well as Wnt- and BMP-inhibitors, failed to maintain *Otx2* expression but the possibility of dilution of the latter two inhibitors by the addition of *XCerS*-expressing cells cannot be ruled out. To clarify the results in this Chapter, more experiments are required using equivalent relative concentrations of cells expressing Wnt- and BMP- antagonists or Wnt-, BMP- and Nodal-antagonists to look at the maintenance of hypoblast-induced markers.

Hypoblast-induced *Otx2* requires both Wnt- and BMP-antagonism to be maintained in the area opaca, unlike *Sox3* or *ERNI*, for which only one or the other is sufficient. This could be because *Otx2* is the only one of the three markers to become restricted to the anterior neurectoderm and mesendoderm during development (Bally-Cuif et al., 1995; Rex et al., 1997; Streit et al., 2000). The maintenance of *Otx2* in the

anterior neurectoderm requires signals from the anterior mesendoderm and it is repressed in the posterior neurectoderm by posterior mesendoderm (Ang et al., 1994; Acampora et al., 1995; Rhinn et al., 1998). *Sox3* is also expressed in the posterior neurectoderm (Rex et al., 1997). The correlation between factors that can maintain expression of the different markers with their normal expression domains suggests that different signals may be involved in neural induction and in forebrain specification.

Sox2 was never induced in any of the experiments in this Chapter. However, *ERNI* expression is maintained by all the combinations of antagonists, along with a hypoblast graft. *ERNI* must be down-regulated for *Sox2* to be expressed in the neural plate (Papanayotou et al., in preparation). A signal to down-regulate *ERNI* might emanate from the node or head mesoderm because it begins to be cleared from the centre of the neural plate outwards starting at stage 4+/5 as the PME emerges and is only observed at the border by stage 6/7 at a stage when the head process is fully extended (Streit et al. 2000). Therefore, some missing signal to down-regulate *ERNI,* whilst maintaining the other markers, might be required for *Sox2* induction.
All of these considerations suggest that a further signal, specific levels or timings of the signals tried, acts after the 'maintenance step' of the revised 'activation-transformation' model (Stern, 2001), is required to neuralise the prospective neurectoderm. What could this signal be? Various combinations of FGFs along with BMP- and Wnt-antagonists were tested in the area opaca for their ability to induce *Sox2* but no induction was ever obtained (Linker and Stern, 2004). I think that the answer might lie in the specific timings of these signals. The experiments with the hypoblast in the previous Chapter show that FGF signalling is required by the hypoblast for *Sox3* expression but that exogenous FGF inhibits *Otx2* induced by the hypoblast and SU5402 up-regulates it. Also, it is possible that there is also an early requirement for RA signalling co-incident and dependent on FGF that is then blocked when *Cyp26A1* is induced. Subsequent antagonism of both Wnt and BMP can maintain the expression of *Otx2, Sox3* and *ERNI* and induce a neural plate-like morphology. FGF and Chordin can maintain *Sox3* but cannot induce a neural plate morphology suggesting that further signals from the hypoblast are required for pre-neural induction. *Cyp26A1* requires additional RA to be maintained. Perhaps at this point, when all the hypoblast-induced factors have been maintained, further

signalling by FGF and RA is required to induce definitive neural tissue. This could be tested by grafting a hypoblast and BMP- and Wnt-antagonist-expressing cells in the area opaca followed by 18 hours' incubation and then the application of beads coated in FGF and/or RA. In this manner, the timing of FGF and RA could be regulated and it might be possible to elicit a definitive neural character.

4.3 Models of A-P patterning and 'Maintenance' Signals

The revised 'activation-transformation' model (Stern, 2001) proposes that the activation step is divided into two: a transient induction of 'pre-neural', 'pre-forebrain' character by the hypoblast and a second 'maintenance' signal that both stabilises genes induced in the epiblast by the hypoblast and to induce a definitive neural, forebrain character. The results from this Chapter show that *Sox3, ERNI* and *Otx2* can indeed be maintained following a hypoblast-mediated induction. *Sox3* and *ERNI* can be maintained by Wnt antagonism, BMP antagonism or a combination of the two. Not only is the induction maintained but the morphology of the area opaca also changes to a thickened neural plate-like structure. *Otx2* is only maintained by combined Wnt and BMP antagonism and it is the only marker of those tested which becomes restricted to the anterior neural plate (Bally-Cuif et al., 1995). The 'two-inhibitor' model (Glinka et al., 1997) for head induction, which proposes a combined inhibition of BMP and Wnt signalling, does not explain this finding because these antagonists alone cannot induce *Otx2* nor *Sox2* and they cannot even induce *Sox2* when combined with a hypoblast.

A mechanism was suggested by Wilson et al. (2000; 2001) whereby an early phase of FGF signalling, in combination with a lack of BMP and Wnt signalling is able to encourage a neural fate. However, the data here (and Linker and Stern, 2004) show that FGF and Wnt- and BMP-inhibition is not sufficient to induce *Sox2* even under conditions that maintain expression of the pre-neural marker, *Sox3.* This suggests again, that other neuralising signals are required.

Furthermore, maintenance of these markers is not sufficient to induce definitive neural character (*Sox2* expression) even when an early neural plate-like morphology is induced. This shows that a further signal is required to induce the expression of *Sox2*. In this respect, the combination of 'activation' and 'maintenance' steps from

the revised 'activation-transformation' model (Stern, 2001) need to be followed by a further, 'neuralising' step to result in a definitive anterior neural state. Alternatively, it is possible that BMP- and Wnt-inhibition are not the normal maintenance factors, and that another signalling pathway(s) capable both of maintenance and *Sox2* induction remains to be found.

In the experiments that led to the 'activation-transformation' model, the region ascribed the greatest activating potential was just caudal to the PME (Nieuwkoop and Nigtevecht, 1954). However, in the chick, the PME emerges from the head mesoderm at stage 5, which could be considered to be the end of neural induction (Dias and Schoenwolf, 1990; Storey et al., 1992).). At stage 5, a node recombined with PME can induce a full secondary axis (Foley et al., 1997) and the head process at stage 6/7 can induce neural tissue in the area opaca (Rowan et al., 1999); however, the epiblast loses competence to be induced at stage 4+, at least in the area opaca (Dias and Schoenwolf, 1990; Storey et al., 1992). Also, *Sox2* is expressed from stage 4+, which marks definitive neural tissue (Rex et al., 1997; Streit et al., 1997). Therefore, neural induction is a process that is likely to start very early on with the hypoblast's induction of the epiblast.

The qualitative models of A-P patterning are based on experiments that show that the axial mesoderm at different levels can induce specific A-P regions of neuraxis (Mangold, 1933) and that the mesoderm emerging from the dorsal lip later induces progressively more posterior neural tissue (Spemannn, 1931; 1938). The PME and head process could be acting to signal vertically to the overlying ectoderm to maintain a 'pre-neural, 'pre-forebrain' state induced by the hypoblast and to impart anterior neural character. The head process had been shown to have different regional inducing abilities at rostral and caudal levels, with the rostral head process inducing neural tissue of forebrain character in the area opaca and the caudal head process inducing markers of a caudal hindbrain/anterior spinal cord character (Rowan et al., 1999). However, some planar signalling might contribute to spread the signals emanating from the mesendoderm through the ectoderm. The neural plate boundary extends much further laterally than the axial mesendoderm; likewise, the area opaca regions that have been exposed to a hypoblast graft followed by chordin-expressing cell pellets expressed *Sox3* in a much broader domain than that directly apposed to the pellet, indicating that the maintenance signals initiated by the ectopic Chordin can spread through the ectoderm.

In summary, the results presented in this and the previous Chapter are most consistent with the revised model of 'activation-transformation' (Stern, 2001). However, although the data show that the transient activation of pre-neural, pre-forebrain markers by the hypoblast ('activation' step) can be maintained by Wnt-, BMP-antagonism and RA ('maintenance' step), this does not induce a stable forebrain state. Further, unknown signals must be required to induce definitive neural character.

Chapter 5: Caudalisation of the neuraxis

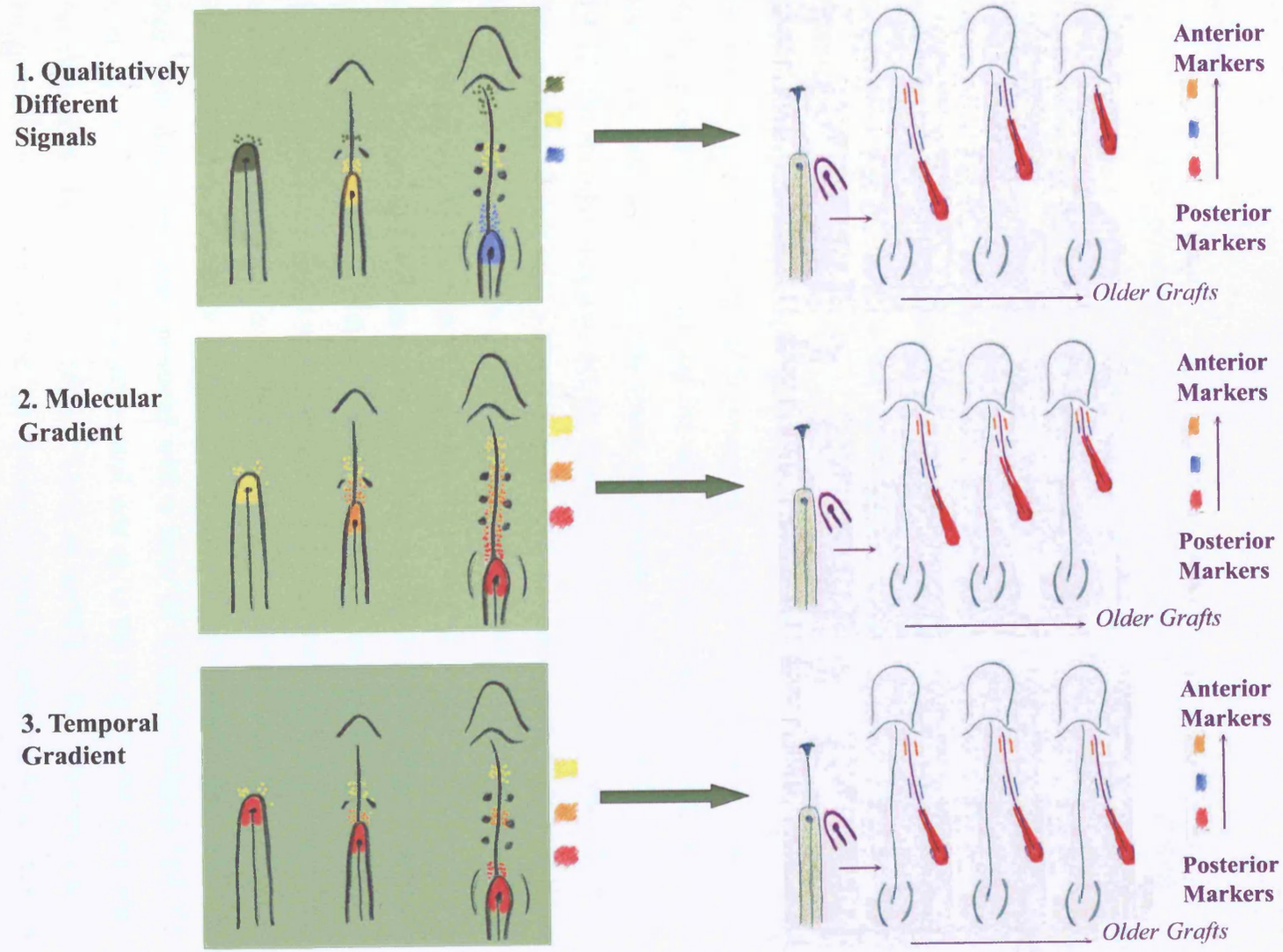
There are several distinct models to account for how caudal regions of the nervous system are generated. In this chapter, some of the features of the models are tested by looking at whether, and if so how, the node can impart regional information to the axis. The experiments take advantage of the observations that, after a certain stage, the avian node is unable to induce a head and also the epiblast becomes unresponsive to inducing signals yet it retains its ability to become regionally specified (Dias and Schoenwolf, 1990; Storey et al., 1992; Foley et al., 1997). In one set of experiments, a node was grafted from different stage donor quails and the secondary axis produced was assessed for A-P markers. The changes in expression of regional markers in the induced axis can help to distinguish between the models by varying the age of donor node used or the amount of grafted material (Fig.5.1). If **qualitatively** different signals are responsible for specifying different regions of the neuraxis (as proposed by Spemannn, 1931; 1938, Mangold, 1933; Holtfreter, 1933), one might expect that the secondary axis induced would progressively lose anterior markers as older nodes are used. There might be a gradient, however (Nieuwkoop and Nigtevecht, 1954); if this is a **molecular gradient**, where caudalising signals become stronger with age, then the more anterior markers in the induced axis should become condensed as older nodes are used. If the gradient is **temporal,** so that the signal remains constant but the time cells spend in its vicinity is important, then the secondary axis should not change.

The pre-somitic region of the embryo has also been suggested to have a patterning influence on the neurectoderm(Muhr et al., 1997; 1999; Liu et al., 2001; Diez del Corral et al., 2002; 2003; Wacker et al., 2004; see Chapter 1). To study the ability of this tissue to provide regional specification and to look at the changes in its caudalising ability with time, pre-somitic mesoderm is homotopically transplanted between different staged donors and hosts and the effect on *Hoxb9* expression in the neural tube is investigated.

The results show that the node has an intrinsic age but that a donor node can be 'reset' by surrounding host tissue to produce a secondary axis of the same A-P character as the host. Pre-somitic mesoderm from younger embryos can anteriorise the neuraxis when grafted into a host whereas that from older embryos can posteriorise the neural tube of the host. These findings suggest that signals from the

presomitic mesoderm pattern the neuraxis and that mesoderm that emerges from the node later can induce more caudal neural regions.

Figure 5.1
Potential Mechanisms of Caudalisation



5.2 Materials & Methods

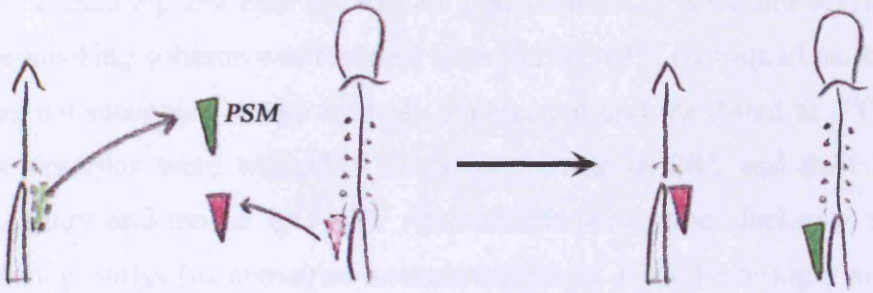
5.2.1 Grafting

Node grafts were performed by removing the quail donor embryo and placing it in Pannett-Compton solution (Pannett and Compton, 1924). The node region was excised using an insect pin. The host embryo was assembled as a modified New culture (New, 1955; Stern and Ireland, 1981). A region of endoderm adjacent to the node of the host embryo was lifted from the mesoderm to form a pocket. The donor node was placed inside this pocket at a slight angle to the host node so that the tips of both nodes were pointing in the same direction. These cultures were incubated overnight.

Node replacement experiments were performed by removing the node from a host chick embryo. The very edge of the area opaca, which does not have any yolky cells, was removed too. This prevents the embryo attaching and stretching across the membrane too quickly and was found to promote healing (Stern & Bachvarova, 1997). The embryo was then set up in a modified New culture (Stern & Ireland 1981). A quail donor node was excised and placed in position in the host to replace the original node. All excess liquid around the embryo and graft was removed, using a pulled glass capillary attached to a mouth pipette, to enable the tissues to knit together. To promote healing further, the cultures were kept at room temperature for two hours before placing in the incubator. The embryos were incubated overnight.

Presomitic mesoderm grafts were performed using the protocol from Stern (1999) for both *in ovo* and operations in embryo culture. Briefly, for modified New culture, the host embryo was placed in Tyrode's-CMF solution containing 0.1% Trypsin in a Petri-dish which had been prepared with a layer of Sylgard (Sylgard 184: Dow Corning). The embryo was pinned, ventral side up, to the Sylgard with insect pins at the edge of the area opaca to stretch it. Using an insect pin, the presomitic region to be removed was scored so that the endodermal and mesodermal layers were cut, leaving the ectoderm intact. The embryo was left for 1-2 minutes for the trypsin to act and then the mesoderm was peeled away from the ectoderm. The mesoderm was discarded and the host transferred to modified New culture. The quail donor was prepared in the same way and the mesoderm removed was placed in the correct

orientation in the hole created by the removal of paraxial mesoderm in the host embryo.



Presomitic mesoderm grafts were also performed *in ovo*. Quail donor embryos were prepared in the same way as above. Host eggs were incubated horizontally. A hypodermic needle was inserted into the blunt end of the egg and 2ml of albumin removed. The eggs were opened by cutting a window (1x1cm) in the shell on the upper-most point, above the embryo. Tyrode's-CMF was added until the embryo floated to the surface. Indian ink (Pelikan Fount India, diluted 1:10 with PBS) was injected beneath the embryo to make it more visible. Silicon grease was deposited around the edges of the window in the shell and the egg topped up with Tyrode's-CMF to form a convex bubble. The vitelline membrane over the region to be operated was nicked and the Tyrode's in the bubble replaced with Tyrode's containing 0.12% (w/v) trypsin (1:250, Difco). The ectoderm overlying the presomitic mesoderm was cut using a micro-ophthalmology blade (micro-feather 15° angled blade made by Feather, distributed by GmbH) following the medial edge of the PSM so that ectoderm and mesoderm were separated from the axial structures. The presomitic mesoderm was carefully removed and the trypsin solution replaced with PBS. The graft from the donor was inserted into the hole and some more albumin extracted with the needle from the original hole. The grease was cleared away and the window sealed with PVC tape after adding 2-3 drops of antibiotic-antimycotic solution (GIBCO, 100x). The egg was inverted and placed horizontally in the incubator. These embryos were cultured for 1-3 days.

5.2.2 QCPN antibody staining

For the older embryos used in this chapter, a hydrogen peroxide (H_2O_2) step was included in the *in situ* protocol whereby embryos were incubated in 6% H_2O_2 (final

concentration in H_2O) for 1 hour before the proteinase K step. Following mRNA *in situ* hybridization, embryos were fixed in 4% PFA. To prepare them for QCPN staining, they were washed 3 times for 1 hour in PBS and then blocked for 1 hour in PBS containing 2% heat inactivated goat serum, 1% BSA and 0.1% Triton X-100. The blocking solution was replaced with 100% QCPN (anti-quail perinuclear antigen: does not recognise chick) antibody supernatant and incubated at 4°C for two days. The embryos were washed 5 times for 1 hour in PBS and then incubated in a secondary anti-mouse IgG-HRP (horseradish peroxidase: Jackson) antibody in the blocking buffer (as above) at a concentration of 1:2000 overnight at 4°C. Embryos were washed again for 5 times for 1 hour in PBS and then rinsed twice in 100mM Tris-HCl, pH7.4. 3,3' diaminobenzdine was added at 200μg/ml and infiltrated for 2 minutes followed by a 1:10,000 dilution of the 30% stock of H_2O_2. Embryos were incubated in the dark at room temperature until the colour had developed (about 10-30 minutes) and the reaction stopped by washing in tap water.

5.3 Results

5.3.1 Testing the ability of Hensen's Node to Caudalise neural plate

5.3.1.1 Generating Secondary Axes Using Different-Staged Donor Nodes

The node loses its ability to induce anterior neural structures once the head process has emerged: when grafted into a host embryo it will give rise to a secondary axis but without generating an ectopic head. The epiblast also loses its ability to respond to inducing signals rapidly after stage 4^+. Instead, cells can be recruited and patterned by a grafted node. This assay was used in the following experiments to test three models of transformation (qualitatively different signals, gradients and time of exposure to a constant signal). Nodes taken from younger or older staged quail embryos were grafted next to the host node of an embryo older than stage 4^+. They generate a secondary axis (by a combination of self-differentiation of the graft and recruitment of host neural plate cells), which lacks a head. The A-P markers expressed by this secondary axis were analysed to investigate which of the models of transformation (discussed in the introduction to this chapter) fit the results best.

The host embryos used were all at stage 5-6. Donor quails were between stage 4^+ (ensuring the head process had emerged) and stage 10. Embryos were grown overnight by which time they reached about stage 9-12. The secondary axis generated was of two forms. It either grew within the plane of the host embryo and was reasonably well extended (22/53)(e.g. Fig.5.2C) or it was attached to the host embryo only at its most anterior point with the remainder growing out of the plane of the host (31/53)(e.g. Fig.5.2B). The results obtained from these two different types of secondary axis were quite different. When the axis grew in the plane of the host, the A-P boundaries of the regional markers analysed were more or less at the same level as their normal expression in the host, regardless of the stage of the donor (22/22) (Fig.5.2C: stage 4^+/5 node grafted into a stage 6 host (9/9). D, F: stage 8 donor node into stage 5-6 host (8/8)). However, when the axis grew out of the plane of the host, it generated an axis with patterns of expression agreeing more with the grafted node than with the host. Representative results are shown in Fig.5.2B, E (Fig.5.2B: host stage 6, donor node stage 4^+/5 (9/9). E: host stage 5, donor node stage 8 (11/11)). In these cases the graft never appeared to influence the patterning of the endogenous axis (1/31) except in one case when the anterior boundary of host *Hoxb4* expression was shifted rostrally (indicating a posteriorisation) on the side of the graft (Fig.5.2A).

5.3.1.2. Replacing the Endogenous Node with a Donor Node from a different stage

The results above suggest that whilst the node does age, signals from the host can respecify the neural plate that develops around the graft. To test this further, nodes were removed from stage 6 hosts and replaced with a node from a stage 8 donor to see whether the new node would caudalise the axis or whether these signals are subordinate to those from the surrounding tissues of the host embryo. Embryos were grown overnight by which time they grew to around stage 13. The latter seems to be the case. As shown in Fig.5.3, the anterior and posterior boundary of expression of neural *Hoxb4* and *Hoxb9* appear to be consistent (4/16) with the positions of the boundaries of control embryos which had received an age-matched donor node, which did not display any clear caudalisation of the axis (n=10). This suggests that an older node can pattern an axis of the age of the host without caudalising it but the results are tentative. However, these embryos did not grow well and it is possible that

in all cases (control and experimental) the axis developed abnormally therefore the results should be interpreted with some caution.

5.3.2 The Role of the Presomitic Mesoderm in Transformation

The above observations suggest that if the node emits patterning signals, these are subordinate to those emitted by host tissues. Which host tissues could be responsible? The presomitic mesoderm has been suggested to play a role in patterning the neurectoderm, as discussed in the introduction (Muhr et al., 1997; 1999; Liu et al., 2001, Diez del Corral et al., 2002; 2003; 2004). Presomitic mesoderm was therefore tested for its ability to provide patterning signals.

5.3.2.1 Grafting PSM from Older Donors into Younger Hosts

The presomitic mesoderm was removed from a host embryo at stage 6-7 or 8 and replaced with that from a quail donor of stage 8-8$^+$ or 9-10 in modified New culture. Similar experiments were done *in ovo* using stage 10-12 hosts and 16-18 somite donors. This experiment allowed assessment of age-related changes in the caudalising abilities of the PSM. When anterior pre-somitic mesoderm (the anterior limit just caudal to the last somite, and the posterior limit at the middle of the segmental plate) was homotopically transplanted, no change in *Hoxb9* expression was observed (0/20). However, when posterior PSM was transplanted (rostral limit at the medial point of the node and extending caudally to the top third – half of the primitive streak) the neural boundary of *Hoxb9* became displaced adjacent to the graft. Fig.5.4 shows the results of these experiments (New Culture embryos fixed after overnight incubation at approximately stage 11, and *in ovo* embryo illustrated at stage 21). A-C (and a-c following QCPN staining) show embryos in which a stage 8$^+$ donor was grafted into a stage 6 host. The anterior boundary of *Hoxb9* expression in the neural tube on the side of the graft is shifted anteriorly, indicating a posteriorisation (10/16). This is also the case when a stage 10 PSM is grafted into a stage 8 host (5/7) or into a stage 7 host (6/9). D shows an embryo of host stage 10 that had received a graft from an 18-somite donor *in ovo*. There is a similar shift in *Hoxb9* (3/5). 16 somite stage PSM could also cause an anterior shift in *Hoxb9* in stage 12 hosts (4/7) and stage 10 hosts (3/7). Whilst there is a consistent posteriorisation of the neurectoderm, the extent varies even between embryos of the

same experimental group, between 1 and 3 somites (an experimental group refers to a set of embryos in which the donors are all the same stage and the hosts are all the same – though different to the donors) and there is not much difference in the extent of the displacement observed between groups. Therefore, it was not obvious if there was a difference in the extent of the caudalisation depending of the stage of the donor relative to the host. The control experiment in which the PSM was replaced with an age-matched donor graft revealed no change in *Hoxb9* boundary (0/15). These results indicate that PSM from older embryos has a stronger caudalising ability on the neuraxis at all stages of donor analysed (between donor stage 8+ to 18 somite stage) and that the host can respond to the change at all stages analysed (stage 6 to 12 somite stage). However, the size of the shift of neural *Hoxb9* does not vary noticeably between the different combinations tried.

5.3.2.2 Grafting PSM from Younger Donors into Older Hosts

The experiment above shows that older PSM has stronger caudalising ability than younger PSM because a graft of PSM into a younger host will caudalise the neuraxis. Can a younger PSM anteriorise the neural tube? The same experiments were performed but this time host stages were 8^+ or 10 and donor stages 6-7 or 8^+ respectively. Following overnight incubation, embryos were fixed at stage 13. This grafting procedure resulted in a posterior shift in the anterior boundary of *Hoxb9* in the neural tube on the side of the graft (Fig.5.5A, B and a, b following QCPN staining) (stage 8+ donor, stage 10 host: 6/9; stage 6/7 donor, stage 10 host: 5/8, stage 6/7 donor, stage 8+ host; 4/6). The extent of the shift was quite consistent (about 1-2 somites) and did not noticeably differ depending on the stages of the hosts and donors. Therefore, younger PSM can anteriorise the neuraxis of an older host but the anteriorisation is not so great as the posteriorisation by older PSM (1-2 somite length of neural tube compared to 1-3 in the reverse operation) and, once again, the different combinations of donor and host stage do not change the extent of anteriorisation.

5.3.2.3 Replacing PSM with Titanium Foil.

To determine whether the posterior shift of *Hoxb9* in the neural tube caused by younger PSM indicates an active anteriorisation (which would be contrary to the

'activation-transformation' model of Nieuwkoop and Nigtevecht, 1954) or rather an absence of posteriorisation (more consistent with the model), the PSM region was removed from a stage 8+ host embryo and replaced with a piece of titanium foil to prevent regeneration of the mesoderm (or filling in from more caudal PSM cells). Embryos were grown overnight by which time they reached stage 11-12. The embryos were analysed for the boundary of *Hoxb9* expression; again there was a posterior shift in the anterior limit in the neural tube on the side of the foil implant (Fig.5.5C, D) (8/12).This shift is within the same magnitude (i.e. 1-2 somites) as when a younger PSM is grafted. This is consistent with the idea that the anteriorisation caused by a younger PSM is due to absence of further caudalising signals.

5.3.2.4 A-P Inversion of the PSM

In the previous experiments in this section, it was noticed that grafts of anterior PSM did not cause a shift in *Hoxb9* expression in the neural tube, while posterior PSM did have an effect. Therefore, the PSM might have different caudalising abilities along its A-P axis. To test this, the entire length of the PSM was removed from a stage 8+ embryo and replaced with that from a stage-matched quail donor but the PSM was inverted along the A-P axis. Embryos were grown overnight by which time they reached stage 11-12. The results show that there is a rostral shift in the anterior neural boundary of *Hoxb9* on the side of the graft (6/10) (Fig.5.5E, F) indicating that there is a difference, within the same piece of PSM, of caudalising ability consistent with the posterior part of the PSM having a stronger ability to caudalise than the anterior part.

Unfortunately the above experiments cannot discriminate between the various models outlined in Chapter 1, but some elements are consistent with each of the models. The qualitative model is backed up by the results in which the ectopic node generates a secondary axis out of the plane of the embryo and is patterned according to the donor's age. Also, the qualitative models suggest that mesoderm emerging from the dorsal blastopore later in development has the ability to induce more caudal regions of the neuraxis (Spemannn, 1931; 1938; Mangold; 1933). Consistently, pre-somitic mesoderm produced later (i.e. that from older donors) can induce more caudal character in the host neural tube.

The quantitative models also have some support from the findings. The secondary axis developing in the plane of the host could indicate a temporal gradient model for A-P patterning. However, this model would assume that the signal stays constant and the time spent in its proximity is important, yet PSM from older embryos can caudalise the neuraxis, indicating a qualitatively or quantitatively different signal with time. It would appear that the younger PSM anteriorises the neuraxis by preventing caudalising signals because similar anteriorisations are observed with a replacement of the PSM with foil and therefore this is consistent with the 'activation-transformation' model (which predicts that posterior regions cannot be anteriorised). There is also evidence of a molecular gradient of caudalising activity within the PSM between anterior and posterior at any given stage, shown by the PSM A-P inversion experiments.

5.3.3 The Role of FGF and Wnt in Transformation

5.3.3.1 Grafts of FGF & SU5402 beads into the PSM region

FGFs have been proposed as transforming factors (Cox et al., 1995; Pownall et al., 1996; Muhr et al., 1997; 1999; Hardcastle et al., 2000; Koshida et al., 2002; Rentzsch et al., 2004 etc.). To test whether FGF can mimic the effects of an older PSM graft, beads coated in 50μg/ml FGF4 or 50μg/ml FGF8 were grafted into the PSM region of a host embryo at stage 8+. Embryos grew overnight and were fixed at around stage 12. Ectopic Hoxb9 expression was induced by FGF4 (6/8) and FGF8 (2/7) when the final position of the bead was anterior to the normal expression domain; the induced expression was always caudal to the bead (8/8) (Fig.5.6A, C). In some instances (2/8 FGF4; 1/7 FGF8), a gradient of Hoxb9 expression was observed with the bead positioned at the anterior point where the expression was weakest (*, Fig.5.5A). When the bead remained within the PSM region, no ectopic expression of Hoxb9 was observed in the neural tube (0/6 FGF4, 0/7 FGF8) but there was a slight anterior shift in the lateral mesoderm expression of *Hoxb9* (FGF4, 2/6; FGF8 1/7) (Fig. 5.5 C, arrow). Also, in these embryos in which the *Hoxb9*-expressing lateral mesoderm was expanded, there was a slight down-regulation of the endogenous mesodermal Hoxb9 expression caudal to the bead (Fig.5.6C *).

When beads (either 1 or 2) of 250mM or 500mM SU5402 were grafted into the same place, no change in neural or mesodermal *Hoxb9* expression was ever observed

(0/20). These results show that FGF can induce ectopic *Hoxb9* but do not reveal a link between increased signalling in the PSM region and an anterior shift in the rostral boundary of *Hoxb9* in the neural tube.

5.3.3.2 Grafts of Dkk1 expressing cells into the PSM region

Wnts have also been implicated in transformation (McGrew et al., 1997; Domingos et al., 2001; Kiecker and Niehrs, 2001; Kudoh et al., 2002; Houart et al., 2002; Nordstrom et al., 2002; Shimizu et al., 2005). To inhibit Wnt signalling, pellets of COS cells secreting Dkk1 were grafted into the PSM region. When Hoxb9 was analysed, the anterior boundary of expression in the neural tube was shifted posteriorly on the side of the cell graft (6/9) (Fig5.6D,E) indicating an anteriorisation. This did not occur when mock transfected cells were used (0/6) (Fig.5.6F). Hence, antagonising Wnt signalling in the PSM region does anteriorise the neuraxis and reinforces the theory that the neural tube is rostralised by inhibiting caudalising signals rather than by changing the identity of posterior neural tissue to a more anterior character, consistent with the 'activation-transformation' model.

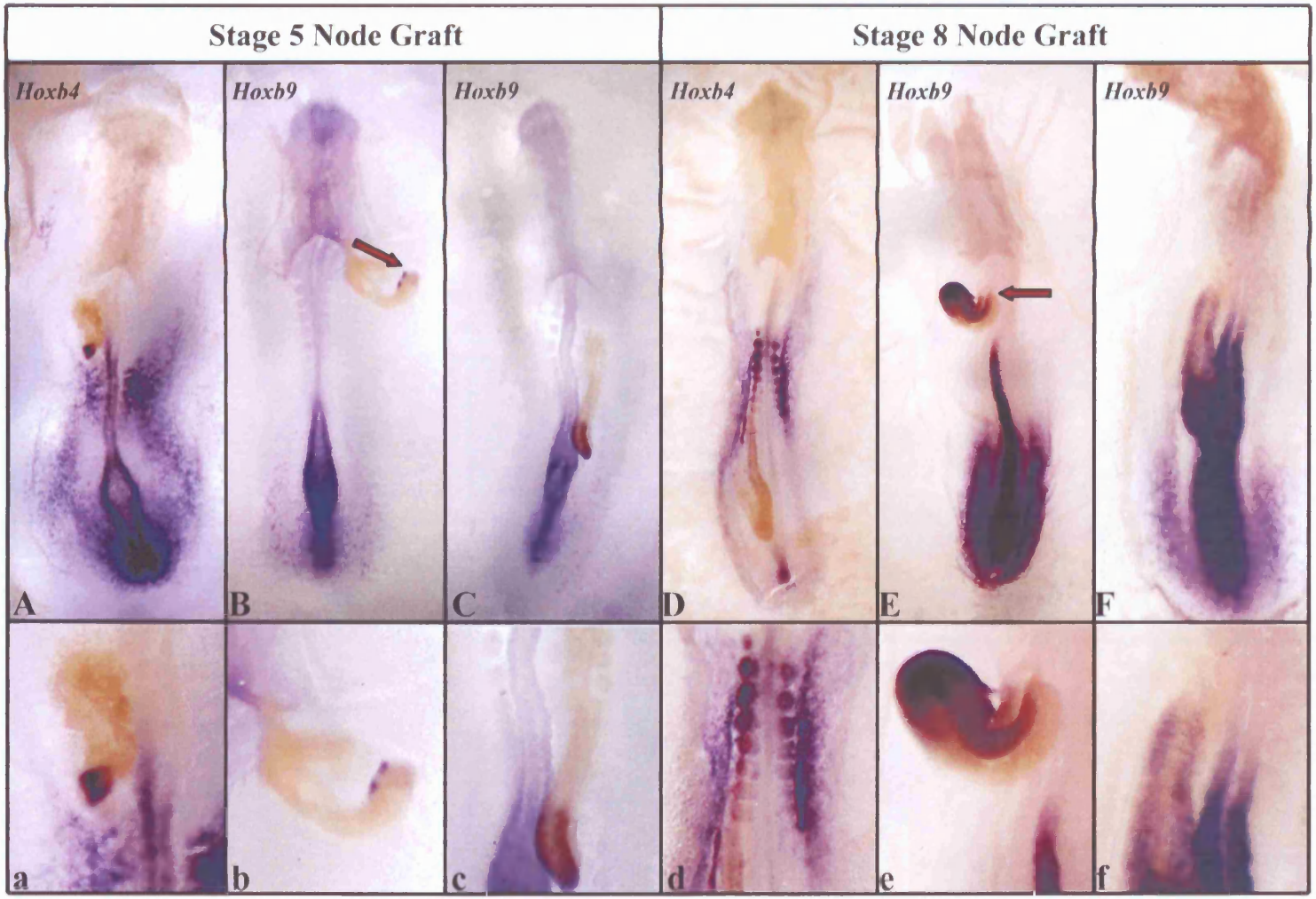


Figure 5.2 When a node is grafted in the prospective hindbrain region of a stage 4 to 6 host two types of secondary axis are produced. This either grows in the plane of the host (C,D,F), in which case it has the same A-P pattern as the host, or it grows out of the plane of the host (B,E) in which case it develops according to the stage of the donor (compare level of *Hoxb9* [arrow] in B with E). QCPN is in brown, *Hoxb9 & Hoxb4* in purple.

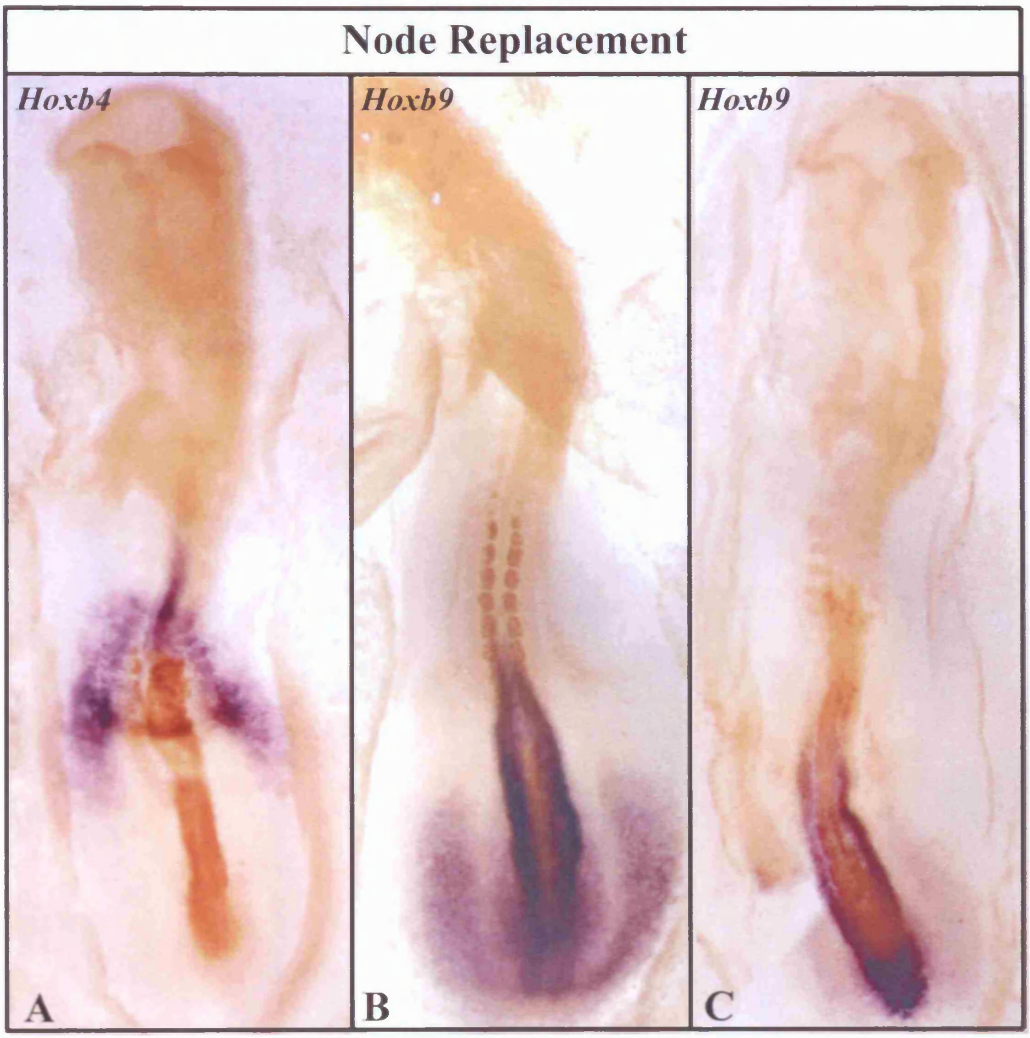


Figure 5.3 Nodes were removed from stage 6 host embryos and repaced with those from stage 8 donors. *Hoxb4 & Hoxb9* are in purple and QCPN is in brown.

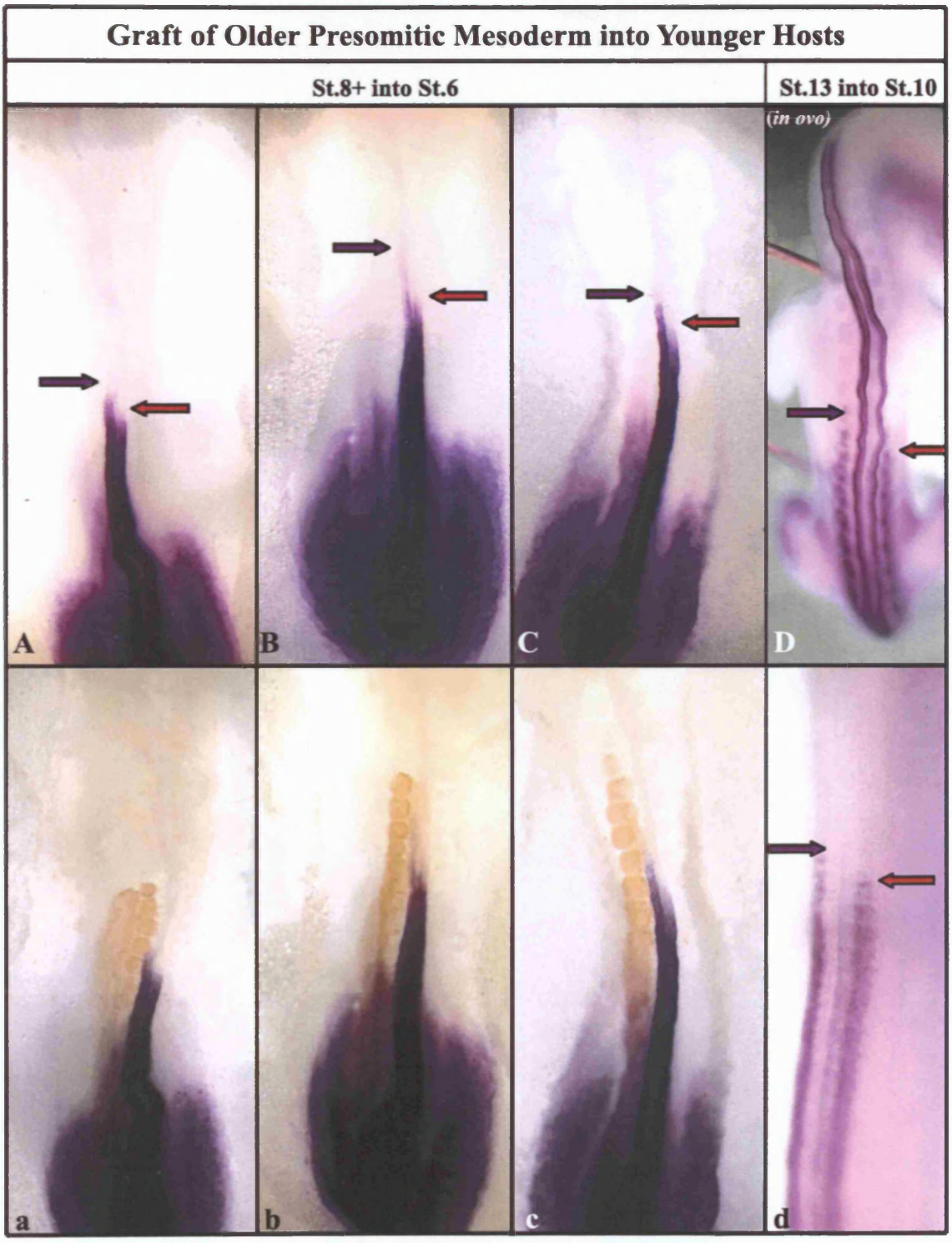


Figure 5.4 Grafting of posterior presomitic mesoderm from an older donor into a younger host. A-C show embryos in which a stage 8+ psm was grafted into a stage 6 host before and after (a-c) QCPN (brown). In D, the psm was from an 18 somite donor and the host was stage 10. Arrows indicate the shift in *Hoxb9* (purple) expression on the side of the graft (blue arrow) and the control contralateral side (red arrow).

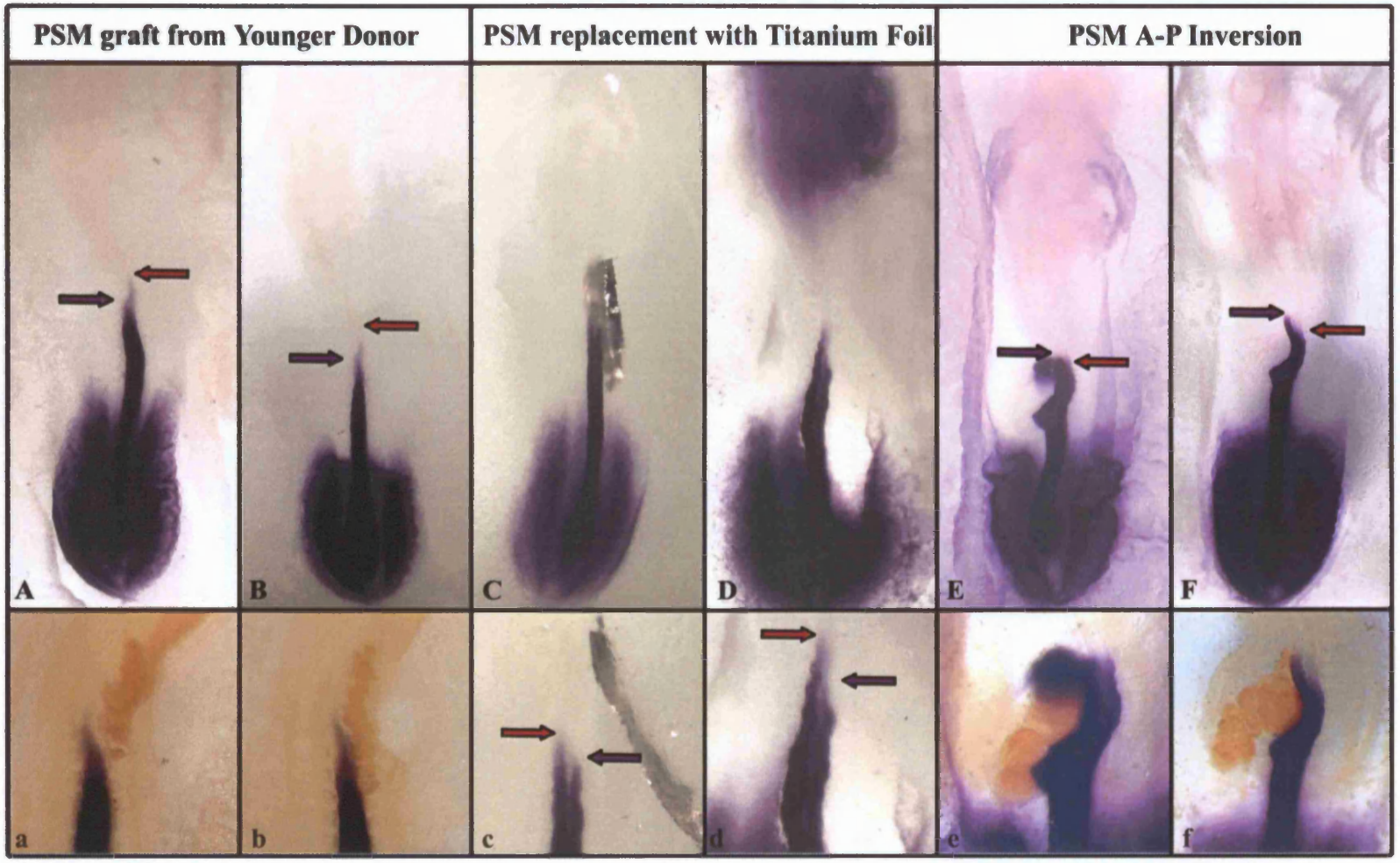


Figure 5.5 Shift in *Hoxb9* expression (purple) following a graft of a stage 7 donor into a stage 8+ host (A-B and a-b), PSM replacement with titanium foil (C-D and c-d), and PSM A-P inversion (E-F and e-f). A-F are dorsal-side views, and a-f are ventral-side views after QCPN staining (brown). Blue arrows indicate *Hoxb9* shift on experimental side and red arrows indicate control side.

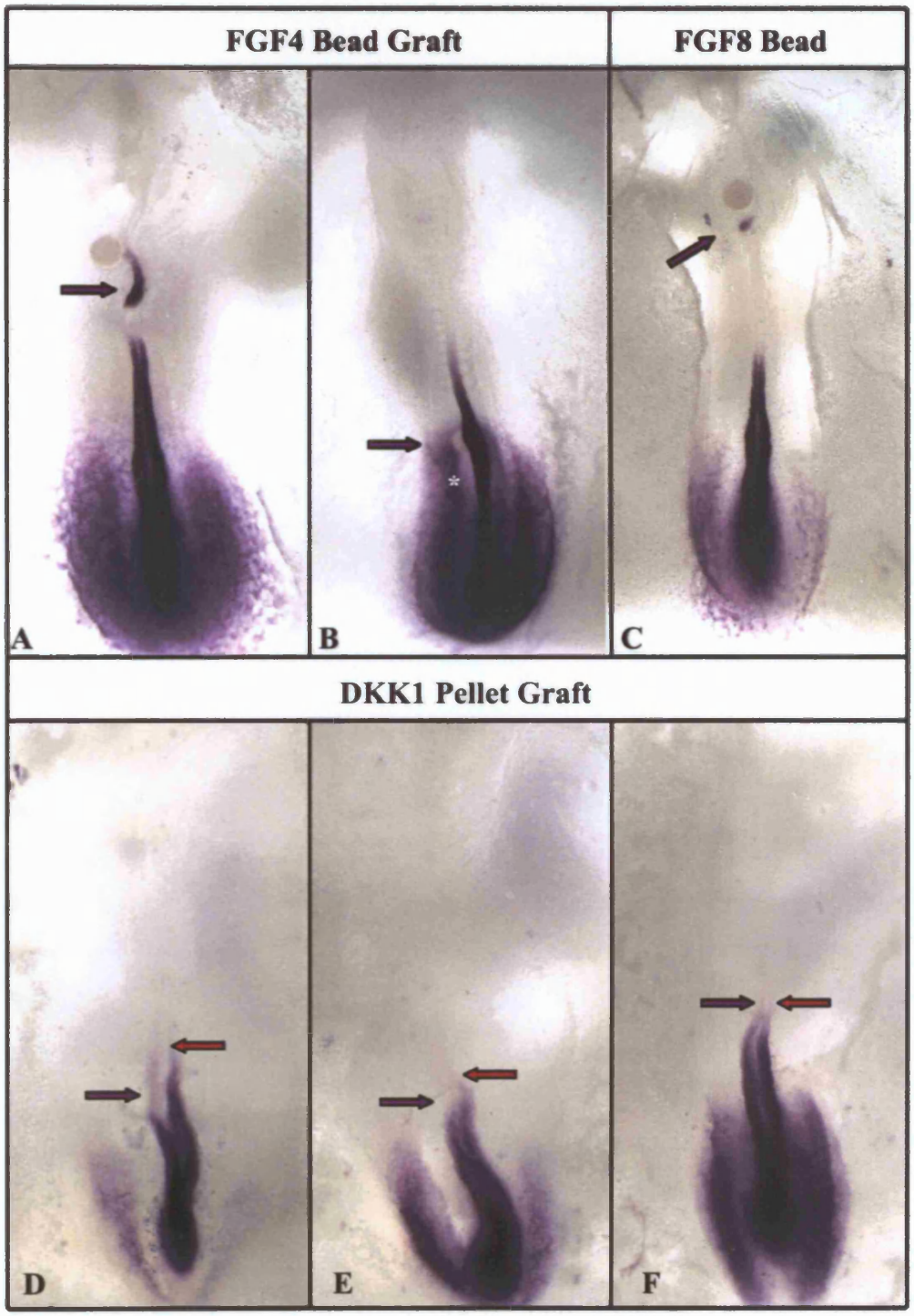


Figure 5.6 An FGF4 bead graft induces *Hoxb9* expression in the neural tube (arrow in A; FGF8 has the same effect, arrow in C), while it can repress it in the PSM (* in B), and cause an anterior shift in lateral mesoderm expression (arrow in B). A graft of DKK1-secreting cells can cause a caudal shift in the expression of *Hoxb9* (blue arrows in D and E; compare to red arrows on ungrafted sides, and to the control F).

5.4 Discussion

i. The Node has an intrinsic 'age' that can be re-set by surrounding tissue

Once induced, the neuraxis is progressively caudalised to establish full anterior-posterior character. Caudalisation has been proposed to occur via two distinct mechanisms: through a gradient (either temporal or molecular) of 'transforming' activity spreading through the ectoderm in the 'activation-transformation' model (Nieuwkoop and Nigtevecht, 1954) or through qualitatively different signals from distinct regions of the adjacent mesoderm in the 'separate organizer' models (Spemannn, 1931; 1938; Mangold, 1933; Holtfreter, 1933). Both models propose that the organizer is important in these patterning events, either because it secretes the transforming signal or because it produces the dorsal mesoderm that will pattern the neural tube. When the organizer retains the precursors of the PME, it can induce or regionalise an axis expressing all rostro-caudal neural markers but after this population of cells leave the node, which occurs at stage 4+ in chick, the remaining node can only induce a neuraxis lacking anterior character (chick: Dias and Schoenwolf, 1990; Storey et al., 1992; *Xenopus*: Schneider and Mercola, 1999; zebrafish: Saúde et al., 2000, mouse: Kinder et al., 2001). These data suggest that the node is involved in A-P patterning but the mechanism is unclear. Therefore, experiments were designed to distinguish between the different models by utilising donor nodes from 4+ onwards and grafting them into the prospective hindbrain region of a stage 5-6 host (this stage epiblast is unable to respond to neural inducing signals, Storey et al., 1992) to generate secondary axes lacking heads. The A-P character of these axes and the way it altered as the donor node was taken from older embryos should provide evidence for either the qualitative or quantitative (temporal or molecular gradient) model (Fig.5.1). Two types of secondary axis developed. When it formed in the plane of the embryo, the A-P pattern of the axis was consistent with that of the host. This could favour the temporal gradient model, the time spent in the vicinity of the node being critical for A-P identity. However when the secondary axis grows out of the plane of the host it develops according to its original stage, indicating that the node does have an intrinsic 'age' for patterning. When the axis grows in the plane of the embryo the node appears to be 're-set' to an age consistent with the surrounding tissues.

The older node is able to induce progressively more posterior Hox genes in explants of neural plate in culture (Liu et al., 2001). For example, a 5 somite stage (ss) node can induce only *Hoxc6* (of the markers analysed) in neural plate whereas a 10ss node can induce *Hoxc6* and *Hoxc9* and a 20ss node can induce only *Hoxc9* and *Hoxc10* but not *Hoxc6*. This effect could be mimicked by adding FGF8 to the neural explants whereby increasing concentrations led to a more posterior neural character being induced (Liu et al., 2001). Therefore although the node loses the ability to induce anterior markers, it can induce a posterior character in neural tissue that is consistent with its age. Hence the node does indeed have an intrinsic age when separated from the embryo, consistent with the experiments in this Chapter when the secondary axis grows out of the plane of the host and is patterned consistently with the node's age at the time of grafting. However, when the organizer and non-organizer mesoderm (NOM) were tested for their relative abilities to induce increasingly posterior neural genes in ectoderm explants with age in *Xenopus*, it was found that the organizer is required to neuralise the ectoderm but cannot impart Hox gene identity whilst the NOM is unable to neuralise but can induce progressively more caudal Hox genes as the NOM ages (Wacker et al., 2004).Wacker et al. (2004) suggest that the organizer is required to provide a constant source of neuralising signal but it is not involved in A-P patterning. Rather, the emerging mesoderm signals vertically to the neurectoderm to give it a regional identity. This mechanism of A-P patterning could apply to the secondary axes produced that grew in the plane of the host. These axes were patterned equivalently to the host and instead of this being due to a temporal gradient, the host mesoderm could be responsible for patterning the secondary axis in a qualitative manner. In summary, the node graft experiments reveal both an intrinsic age that is maintained by the node when separated from surrounding tissues, suggesting a timing mechanism for specification of the neuraxis, although the node also appears to be sensitive to signals from surrounding tissues.

ii. The Posterior Pre-Somitic Mesoderm has a role in A-P patterning

The node graft experiments suggest a role of the surrounding tissues in patterning the neural tube. The paraxial mesoderm has been implicated in A-P specification of the neuraxis (Itasaki et al., 1996; Muhr et al., 1997; 1999; Gould et al., 1998; Diez del Corral et al., 2002; 2003) and the anterior pre-somitic mesoderm (PSM) was shown

to be able to caudalise explants of neural plate increasingly as it was taken from older donors (Muhr et al., 1997; 1999). Therefore, experiments were performed to assess the role of the PSM in A-P patterning. Grafts of either anterior or posterior PSM were placed homotopically in either younger or older hosts to assess the effect on the anterior boundary of *Hoxb9* in the neuraxis. Anterior PSM did not affect *Hoxb9*, which is curious because Muhr et al., (1997; 1999) did see an effect with this tissue. However, posterior PSM transplants could affect the neural boundary of Hoxb9: an anterior shift was observed by grafting older PSM and a posterior shift after grafting younger PSM. The reason why the posterior PSM, rather than the anterior PSM causes such a shift could be due to the neurectoderm at the level of anterior PSM being less responsive to changes in caudalising signals than the caudal neural plate at the level of the posterior PSM. Alternatively it could be due to the gradient of *FGF8* in the PSM that decreases towards the anterior (Dubrulle et al., 2004). *FGF8* is not expressed in the anterior PSM; in this region, *RALDH2* is detected and it has been shown that RA both represses the expression of *FGF8* and causes cells to leave the caudal neural plate and differentiate (Diez del Corral et al., 2002; 2003) whilst FGF signalling in the posterior PSM has been shown to maintain neural progenitors in the caudal neural plate and delay neural differentiation (Mathis et al., 2001; Diez del Corral 2002). Therefore, perhaps the posterior PSM has a stronger caudalising influence than the anterior PSM because it has a higher level of *FGF8*. Indeed, when the entire length of the PSM was rotated about its A-P axis, the neural tube at the level of the posterior PSM (now in the rostral position) is caudalised indicating that within the PSM there is a varying posteriorising ability that is greatest most caudally.

It has been suggested that the role of FGF signalling in the PSM is to maintain neural progenitors in the caudal neural plate and thus cause them to have a more posterior identity (either by a temporal mechanism whereby the longer they stay in contact with a constant signal, the more caudal their character, or by keeping them in the vicinity of a qualitatively or quantitatively changing signal) (Mathis et al., 2001; Diez del Corral et al., 2002; 2003). However, this does not account for a change in caudalising ability of the posterior PSM taken from differently aged donors. Posterior PSM from a stage 10 donor can caudalises the neural tube of a stage 7 host suggesting that PSM from an older embryo produces a stronger caudalising signal than PSM from a younger embryo. However, in these experiments, a difference in

the length of the shift in *Hoxb9* was not observed between, for example, when a stage 10 donor PSM was grafted into a stage 7 host compared to a when a stage 8+ donor PSM was grafted. This interpretation is confounded by variable shifts even within once specific stage of donor and host. Furthermore, it is possible that the embryo recovers from an initial shift in *Hoxb9* to revert to a normal expression by subsequent instruction from host mesoderm and so a difference depending on the age of the donor might have been missed. However, in the PSM experiments, the graft differentiates into somitic mesoderm, staying in contact with the neuraxis, which might allow it to reinforce the patterning signals. The graft-derived paraxial mesoderm does have a more anterior boundary of *Hoxb9* expression compared to the contralateral side although the anterior region that has differentiated into somites was not tested for Hox identity and therefore it is unclear whether this becomes re-specified to the A-P character of the adjacent host neural tissue. Despite this, the PSM from older embryos is consistently able to caudalise the neuraxis of younger embryos suggesting that there is a difference in the type, or strength, of signal that emanates from it. Although Muhr et al. (1999) analysed rostral PSM, they also found that PSM taken from older embryos has a stronger caudalising ability than that taken from younger embryos, which can be mimicked by increasing concentrations of FGF (Liu et al., 2001). Therefore, these data, along with the results presented in this Chapter, suggest that there is a change in the signals emanating from the PSM as the embryo ages and that they become more caudalising.

iii. Anteriorisation of the neuraxis by younger posterior PSM might act by preventing further caudalising signals.

Nieuwkoop's model of 'activation-transformation' specifies that once tissue is 'activated' to have an anterior, neural character, it is subsequently transformed to different extents to produce a fully patterned rostrocaudal axis (Nieuwkoop and Nigtevecht, 1954). Therefore according to this model, it is not possible to anteriorise tissue but only posteriorise it. In the experiments in which PSM derived from a younger donor is grafted into an older host, the neuraxis is 'anteriorised'. Hence, it is important to distinguish between a direct anteriorisation of the neural tube and prevention of further posteriorisation by removing the source of caudalising signals. An experiment to disentangle these two possibilities was performed whereby the PSM was removed and replaced with titanium foil (to prevent regeneration). These

results showed that the anterior boundary of *Hoxb9* was shifted caudally, to a similar extent (1-2 somites) to that of younger PSM. Furthermore, when cell pellets secreting the Wnt antagonist, Dkk1are grafted into the posterior PSM region of an embryo, a caudal shift in *Hoxb9* expression is observed. This indicates that by suppressing a 'caudalising signal' (discussed below), the axis is anteriorised. Foley et al. (1997) showed that the PME, when grafted into the prospective hindbrain region of a host embryo, can change the fate of these cells to forebrain and hence anteriorise them. The PME might be acting to antagonise posteriorising signals like Wnts, which have been shown to act in a concentration-dependent way to caudalise the brain (Kiecker et al., 2001: Nordstrom et al., 2002) by secreting Wnt antagonists (Chapman et al., 2004; also Dkk1 in the PME has been shown to be important in specifying the anterior neurectoderm in mouse: del Barrantes et al., 2003 and *Xenopus*, Glinka et al., 1998; Kazanskaya et al., 2000). This is not the same as converting tissue that is already committed to a posterior fate, to become anterior. However, Dasen et al. (2003) showed that *Hoxc6*, electroporated into the chick neural tube into post-mitotic, thoracic regions, can repress *Hoxc9* as well as the other way around suggesting that it is possible to change a posteriorly specified region to a more anterior fate. In summary, the majority of the evidence is in favour of the proposal of progressive caudalisation in the 'activation-transformation' model, but there are also some data arguing against it.

iv. The molecular nature of the caudalising signals produced by the PSM

Wnts, FGFs and RA have all been shown to act to caudalise the neuraxis (FGF: Cox et al., 1995; Pownall et al., 1996; Muhr et al., 1997; 1999; Hardcastle et al., 2000; Koshida et al., 2002; Rentzsch et al., 2004 etc., RA: Blumberg et al., 1997; Chen et al., 2001; Dope and Lumsden, 2001; Kaiser et al., 2003; Shiotsugu et al., 2004; Molotkova et al., 2005 etc., Wnt: McGrew et al., 1997; Domingos et al., 2001; Kiecker and Niehrs, 2001; Kudoh et al., 2002; Houart et al., 2002; Nordstrom et al., 2002; Shimizu et al., 2005). Wnts have been shown to be able to caudalise the neurectoderm directly, in a concentration-dependent manner without first inducing mesoderm (McGrew et al., 1997; Kiecker and Niehrs, 2001; Domingos et al., 2001; Nordstrom et al., 2002) and this caudalisation is dependent on the presence of FGF signalling (McGrew et al., 1997; Domingos et al., 2001; Nordstrom et al., 2002). FGFs have been shown to posteriorise the neuraxis in a concentration-dependent way

(Lamb and Harland, 1995; Kengaku and Okamoto, 1995; Liu et al., 2001) and different FGFs are thought to act directly on the neurectoderm, as is the case for FGF8 (Hardcastle et al., 2000), or might indirectly via the generation of mesoderm (Muhr et al., 1999) expressing downstream targets Cdx and Hox genes in the case of FGF4/eFGF (Pownall et al., 1996). RA is thought to act via the mesoderm to impart specific rostro-caudal identity to the neuraxis (in zebrafish: Begemann et al., 2001; and mouse: Molotkova et al., 2005).

Caudal genes are a convergence point for RA, FGF and Wnt signalling pathways: each has been shown to regulate cdx induction or expression to affect posterior patterning (reviewed in Lohnes, 2003; also Prinos et al., 2001; Houle et al., 2003; Shiotsugu et al., 2004; Shimizu et al., 2005).

The PSM can caudalise the neuraxis (results presented here and Muhr et al., 1997; 1999; Wacker et al., 2004; Diez del Corral et al., 2002). The results from this Chapter hint at a role for FGF and Wnt in this process. There are other data that suggest a molecular mechanism by which signalling in the PSM can pattern the neural tube. Liu et al. (2001) suggested that some signals in the PSM, like FGF, are involved at all stages and act in a gradient to induce more posterior character in neurectoderm at higher concentrations whereas other signals only act at specific times of development, for example GDF11 in the PSM at 14-15ss, which works in concert with FGF at later stages to induce more posterior character in the neuraxis and, conversely, RA acts at younger stages (5ss), when there is little FGF, to activate more rostral Hox genes. This would agree with a qualitative model of A-P patterning whereby distinct signals, or specific combinations of signals, act to specify a particular regional identity in the overlying neurectoderm. FGF8 does not just have an effect on specifying A-P character of the cells in the caudal neural plate; it has also been shown to induce more posterior Hox genes when over-expressed in the neural tube by electroporation (Dasen et al., 2003), consistent with the results in this thesis in which FGF-soaked beads can induce ectopic expression of *Hoxb9* in rostral neural tube. This suggests a continued role for FGF in refining the pattern of the neural tube after it has been initially specified.

FGF signalling by the PSM could act in a concentration-dependent way to caudalise the neuraxis (Liu et al., 2001) but it has also been shown that there is a mutual repression of FGF and RA at the boundary between anterior and posterior PSM, with RA encouraging neural differentiation in the anterior whilst FGF in the posterior

PSM maintains neural progenitors in the caudal neural plate (Diez del Coral et al., 2002; 2003) and this could act to keep these neural progenitors in an unspecified state in the vicinity of further caudalising signals (Mathis et al., 2001). Hence, FGF in the PSM may have two distinct roles for caudalising the neuraxis. Firstly, it acts via the posterior PSM to delay cells leaving the caudal neural plate (Diez del Corral et al., 2002) and secondly, it 'transforms' them into a progressively caudal character as its concentration increases with the age of the embryo (Liu et al., 2001; Dasen et al., 2003).

v. Models for A-P patterning and the caudalisation of the axis.

The caudalisation of the axis could be achieved in different ways. In the 'qualitative' models, each region of mesoderm produces a distinct signal to impart specific antero-posterior character to the overlying ectoderm. The 'activation-transformation' model suggests instead that there is a gradient of transforming signal, increasing towards the posterior end of the embryo, and that this spreads through the ectoderm to caudalises the neuraxis. These two models are intrinsically incompatible but that does not rule out the possibility that both planar and vertical signals pattern the neurectoderm. Equally, just because there is vertical signalling this does not rule out a gradient of caudalising activity as opposed to distinct signals to pattern each A-P position.

Looking at results in favour of the 'activation-transformation' model, the present PSM transplantation experiments show that firstly, the caudalising signal becomes stronger in older embryos, indicating a gradient of transforming ability and secondly, within the PSM at any given stage, the younger, more posterior regions have a greater ability to transform: two assumptions of this model. Furthermore, younger PSM does not seem able to anteriorise caudal neurectoderm but rather prevents it from receiving further caudalising signals. However, the signals that emanate from the PSM presumably pass vertically and it is unclear whether there is any component of planar spreading through the ectoderm. The node graft experiments in which the secondary axis grows in the plane of the host could be indicative of a temporal gradient of caudalising activity in which it is the time spent in the proximity of caudalising signals that specifies A-P identity (Mathis et al., 2001). This has been shown to be involved in maintaining progenitor cells in the caudal neural plate (Diez del Corral et al., 2002; 2003). As the only mechanism of caudalisation, a temporal

gradient would seem irreconcilable with the secondary axes that develop out of the plane of the host, which suggest that the node has an intrinsic age that manifests itself by the formation of an axis with more posterior character the older the donor node (also shown by Liu et al., 2001). This ability has been shown in *Xenopus* non-organizer mesoderm and the mechanism suggested was that a Hox gene clock governs the A-P co-ordinate of the axis (Wacker et al., 2004). However, the secondary axes that develop out of the plane of the host are self differentiated (as shown by co-expression of QCPN and *Hoxb4/Hoxb9*) and could therefore be patterning graft-derived neurectoderm. If this is the case, the donor neural progenitors will have already spent longer in the vicinity of the node than the neurectoderm of the host and will consequently differentiate in a pattern consistent with the donor age.

The qualitative models for A-P patterning are compatible with a timing mechanism. Node-derived notochord or pre-somitic mesoderm (Selleck and Stern, 1992; Psychoyos and Stern, 1996) in the secondary axes could vertically pattern the overlying ectoderm and this pattern would depend on the age of the donor. As the node is taken from a progressively older donor, it would only produce mesoderm to specifically induce in the neurectoderm the A-P identity just caudal to that last formed in the donor embryo. This would explain the results in which the secondary axis grows out of the plane of the host and it is consistent with Mangold's (1933) experiments in which different axial levels of invaginated archenteron roof can specify distinct regions of neuraxis. It is also consistent with the experiments of Spemannn (1931; 1938), which revealed that mesoderm emerging from the dorsal lip later will induce more posterior structures. The experiments in which the secondary axis develops in the plane of the host can be reconciled with this model by ascribing the role of vertical signalling to the host mesoderm or by imagining that the donor node is reset to the same timing as the host. The PSM, when homotopically transplanted also reveals that mesoderm emerging from the node at different stages has different caudalising abilities. However, there is no evidence in the results in this Chapter that the signals emanating from the PSM are qualitatively different at different stages. Instead, the shift in *Hoxb9* expression in the neural tube does not change dramatically when different staged donors are used (there is variability in the shift at all stages analysed), which might be expected if qualitatively different signals were produced to pattern each part of the neuraxis. However, as Sala (1955) pointed

out, there could be various gradients of signal in the mesoderm with one region having different combinations to another and that could account for a specific regional character given to the overlying neurectoderm. If this factor is taken into account, then the results presented in this Chapter are also consistent with a qualitative model in which signalling from the mesoderm that emerges from the node with different properties, as the embryo ages, to enable it to define a region of A-P character.

Chapter 6 General Discussion

6.1 Signals involved in A-P patterning

The results presented in this thesis implicate RA and FGF signalling in the transient induction of pre-neural, pre-forebrain markers by the hypoblast. This is consistent with previous reports suggesting their involvement in early anterior specification (Knezevic and Mackem, 2001; Halilagic et al., 2003; Shiotsugu et al., 2004; Wilson et al., 2000; Delaune et al., 2005; Khokha et al., 2005). Other studies have suggested an early requirement for Wnt signalling for neural induction (Bainter et al., 2001; Baker et al., 1999; Sokol et al., 1995; Wessely et al., 2001; Kuroda et al., 2004) but the present study did not obtain supporting evidence for such a role.

It is also shown here that markers transiently induced by the hypoblast can be maintained by BMP- and Wnt-antagonists (*Sox3, ERNI, Otx2*) and RA (*Cyp26A1*). This supports other findings that inhibition of BMP and Wnt signals is required for head development (Glinka et al., 1997; 1998; Bachiller et al., 2000; Wilson et al., 2001; Anderson et al., 2002; del Barco Barrantes et al., 2003).

FGF and Wnt have also been shown here to act as caudalising factors on the neuraxis. Other studies have illustrated such a role for FGF, Wnt and RA (FGF: Cox et al., 1995; Pownall et al., 1996; Muhr et al., 1997; 1999; Hardcastle et al., 2000; Koshida et al., 2002; Rentzsch et al., 2004 etc., RA: Blumberg et al., 1997; Chen et al., 2001; Dupe and Lumsden, 2001; Kaiser et al., 2003; Shiotsugu et al., 2004; Molotkova et al., 2005 etc., Wnt: McGrew et al., 1997; Domingos et al., 2001; Kiecker and Niehrs, 2001; Kudoh et al., 2002; Nordstrom et al., 2002; Shimizu et al., 2005). Thus, interestingly, these three signalling pathways appear to be involved in both the initial 'activation' step and in the final 'transformation' of the generation of the early neural plate. If the signals responsible for activation are the same as those underlying transformation, cellular context, developmental history of the cells and the timing of the signals must be critical in eliciting the correct responses at the appropriate time.

6.2 Relating the results to models of A-P patterning

6.2.1 Quantitative Models

The 'activation-transformation' model (Nieuwkoop and Nigtevecht, 1954) proposed that an activation signal, emanating from tissue just caudal to the PME, induces anterior neural character in the neighbouring ectoderm. Some parts of this induced anterior neurectoderm is then transformed to yield more caudal fates by a gradient of caudalising activity that increases posteriorly. The transformation signal was proposed to spread in the plane of the ectoderm.

The results obtained in this thesis suggest that the initial activation step occurs much earlier in development, long before the appearance of PME and other mesoderm. It had already been suggested that neural induction probably ends at stage 4+, when the PME emerges from the tip of the node, when the first definitive neural marker, *Sox2,* is first expressed, when the node starts to lose its ability to induce neural tissue and when the area opaca epiblast loses competence to respond to neural inducing signals (Dias and Schoenwolf; Storey et al., 1992; Rex et al., 1997). The hypoblast can induce a pre-neural, pre-forebrain character in the area opaca (this thesis and Foley et al., 2000; Streit et al., 2000). In the embryo, *Sox3, ERNI, Otx2* and *Cyp26A1* are first expressed in the epiblast prior to streak formation, and the hypoblast is able to induce all of them. These findings suggest that neural induction and anterior neural specification commence with the transient induction of these markers by the hypoblast at pre-streak stages. Therefore, the activation step of the model appears to occur much earlier than proposed by Nieuwkoop and Nigtevecht (1954), at least in the chick.

An alternative, the 'double potency' model incorporates both a molecular signal and morphogenetic movements to explain embryo patterning (Yamada, 1940; 1950). Morphogenetic movements direct cells towards or away from a steady signal and regulates the time the cells spend in the vicinity of this signal. Although the experiments here did not test the role of the hypoblast in influencing cell movements, previous work has shown that it does direct the movements of the prospective forebrain (Waddington, 1930; 1932; Foley et al., 2000). In mouse, indirect evidence for this comes from the *Cripto* mutant in which the AVE remains at the distal tip of the embryo (Ding et al., 1998). In these mutants, the forebrain is specified but also remains in a distal position, adjacent to the AVE. Therefore, the movement of the

prospective forebrain to the anterior pole is directly or indirectly dependent on the movement of the AVE. These data suggest that morphogenetic movements are required for correct specification of the axis and support this aspect of the double-potency model.

Other "quantitative" models, including the 'activation-transformation' model (Nieuwkoop and Nigtevecht, 1954), propose that the caudalisation of the neuraxis occurs by means of a posteriorising gradient. This gradient could be a temporal one, in which the time cells reside in the vicinity of a steady signal results in them acquiring a specific A-P character, or of signal strength, in which the caudalising signal increases to generate a more posterior neuraxis. The data presented here could support a temporal gradient. Secondary axes generated in the plane of host embryos are patterned equivalently to the host.

The PSM has been proposed to be involved in the specification and maintenance of a 'stem zone' of neural progenitors in the caudal neural plate (Diez del Corral et al., 2002; 2003; Delfino-Machin et al., 2005). The maintenance of progenitors in this zone would expose them to caudalising signals for longer (Mathis et al., 2001; Diez del Corral et al., 2004; Delfino-Machin et al., 2005). However, there might also be a gradient of signal strength. In Chapter 5, PSM taken from older embryos can caudalise the neural tube when grafted homotopically into younger hosts. This suggests that both an increase in signal strength and a temporal gradient might act to posteriorise the neuraxis.

6.2.2 Qualitative models

"Qualitative" models propose that molecularly distinct vertical signals emanating from the underlying mesoderm induce specific A-P regional character in the neurectoderm (Spemannn, 1931; 1938; Mangold, 1933; Holtfreter, 1933; 1936; Itasaki et al., 1996; Poznanski and Keller, 1997; Agathon et al., 2003; Kudoh et al., 2004; Wacker et al., 2004).

The data presented in this thesis are consistent with the concept that the extraembryonic endoderm in chick does not act as a 'head organizer' (Acampora et al., 1998; Rhinn et al., 1998; Liu et al., 1999; Tam and Steiner, 1999; Foley et al., 2000). The hypoblast induces pre-neural, pre-forebrain character only transiently and cannot induce definitive neural character (this thesis; Knoetgen et al., 1999; Foley et

al., 2000). Maintenance of markers transiently induced by the hypoblast still does not result in neural induction. Therefore, despite the hypoblast's early role in anterior neural specification, it cannot be described as an organizer.

There is evidence, contrary to the concept of an 'activation' step, that neural tissue can be induced that is posterior in character, in the absence of prior induction of an anterior neural state: noted even by Nieuwkoop himself (Mangold, 1933; Nieuwkoop and Nigtevecht, 1954; Storey et al., 1992; Storey et al., 1998; Agathon et al., 2003; Wacker et al., 2004). It is proposed that the signal responsible is mediated by the PSM at late primitive streak stages in part by FGF and it acts to specify the 'stem cell zone' of neural progenitors in the caudal neural plate (as mentioned above: Storey et al., 1998; Diez del Corral et al., 2002; 2003; Delfino-Machin et al., 2005). This is incompatible with the 'activation-transformation' model and indicates a different mechanism is acting to pattern posterior neural tissue that does not require a preceding anterior neural state.

The data obtained here on caudalisation of the neuraxis do not entirely rule out a qualitative model. Secondary axes generated out of the plane of host embryos are patterned according to the donor age suggesting an intrinsic timing mechanism but could also be consistent with a changing system of molecular signals generating progressively more caudal character. Additionally, PSM taken from older donors can caudalise the neuraxis of younger hosts. This suggests that the signals from the PSM change as the embryo ages but does not distinguish between a qualitative or quantitative change.

The concept of a distinct tail organizer, residing in the ventral margin in the zebrafish, has also been proposed (Agathon et al., 2003). This tissue can generate ectopic tails when grafted into the animal pole of a host embryo: an ability that can be mimicked by triple overexpression of Wnt, BMP and Nodal (Agathon et al., 2003) and therefore these signalling pathways, along with FGF (Kudoh et al., 2004) potentially converge in tail development. However, it is not clear whether the tissue grafted by Agathon et al. (2003) can be described as a true organizer because the distinction between recruitment of host cells and induction was not analysed.

In summary, the results obtained here are consistent with aspects of all of the models proposed for A-P patterning. However, they fit best with the revised model of 'activation-transformation' (Stern, 2001). There is a transient activation of a pre-

neural, pre-forebrain character induced by the hypoblast. This can be maintained by additional signals, potentially from the PME, head process and/or ADE. Unlike the modified Nieuwkoop model, however, it appears that maintenance is not sufficient for the induction of a definitive neural state; therefore either an additional neuralising step is required, or the endogenous signals responsible for both maintenance and definitive neuralisation remain to be identified. The neuraxis is subsequently transformed although the precise mechanism for this transformation is still unknown.

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